

PHYSIOLOGICAL STUDIES OF THROMBOSPONDIN

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## ABSTRACT

Thrombospondin (TSP), a glycoprotein of wide cellular distribution, exhibits several activities important in platelet aggregation, haemostasis and cell adhesion. The aim of this project was to investigate the production and role of TSP in human tissues and fluids; particularly its source in the breast and relation to malignant disease.

Most circulating TSP was contained within platelet  $\alpha$ -granules and released upon activation of these cells. Infused TSP appeared to be rapidly bound, while its clearance from the circulation was relatively slow. Extra-platelet sources contributed substantially to basal plasma levels; platelet-associated TSP may therefore be a better indicator of platelet activation than plasma concentration.

TSP concentration in breast cyst fluids varied according to cyst type and correlated inversely with epidermal growth factor. TSP was present in milk at high levels compared with plasma and its pattern of secretion resembled that of IgA; it may be that white cells which infiltrate the mammary gland are a major source of TSP in breast secretions.

Very high levels of TSP were found in malignant breast tissue compared with non-malignant breast and were associated with the centre of the tumour mass. Positive correlation between TSP and von Willebrand factor suggested that endothelium contributes to the high levels of both proteins in malignant breast, whereas lack of correlation between TSP and tissue plasminogen activator argued

against epithelium being the source of TSP in breast cancer.

Studies of TSP in cultured cells confirmed and extended knowledge of its ubiquitous nature. Factors which were important in determining the amount of TSP produced in culture were; cell type, tissue origin, cell density and composition of growth medium, but all cells secreted TSP in a linear, continuous manner. Serum, but not TSP, appeared to be important for maintenance of adhesion and normal morphology of endothelial cells. Production of TSP by breast cancer cells was stimulated by sex hormones and inhibited by corticosteroid, while insulin had a marked effect on TSP secretion.

Monoclonal antibodies generated against calcium-deplete TSP were primarily IgM and gave unique binding patterns in immunoassays, suggesting that they were directed against different epitopes of TSP.

Immunocytochemical localisation of TSP showed a restricted distribution in tissues and cells. Strong staining of infiltrating cells in breast cancer stroma and in germinal centres of tonsil was evidence that TSP may be involved in the immune response.

In conclusion, the results presented in this thesis support current knowledge of TSP as an adhesive glycoprotein of platelets, vessel walls and connective tissues. Novel studies in the breast have revealed a marked association of TSP with cancerous tissue and some cyst fluids, which could be due to production by vascular endothelium and certain white blood cells. While the role of TSP in the breast remains to be defined, its relation to disease states may be of particular physiological significance.

## DEDICATION

To my mother and father, Nancy and Ken, for their love and support throughout all my studies.



## DECLARATION

I hereby declare that this thesis has been composed solely by myself and the work described herein has been my own, except for the contributions of others where indicated.

David A. Pratt

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## LIST OF ABBREVIATIONS

ACD	acid-citrate-dextrose
ADP	adenosine diphosphate
ATIII	antithrombin III
BSA	bovine serum albumin
BTG	beta-thromboglobulin
cDNA	'complementary' deoxyribonucleic acid
CPD	citrate-phosphate-dextrose
DAB	3,3'-diaminobenzidine tetrahydrochloride
DHA	dehydroepiandrosterone
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulphoxide
ECGS	endothelial cell growth supplement
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbant assay
ER	oestrogen receptor
ESR	electron spin resonance
ETP	EDTA-theophylline-prostaglandin E <sub>1</sub>
Fg	fibrinogen
FPLC	fast protein liquid chromatography
Gp	glycoprotein
HAT	hypoxanthine-aminopterin-thymidine
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
HOF	human oral fibroblasts
HRGP	histidine-rich glycoprotein
HSF	human skin fibroblasts
HSVEC	human saphenous vein endothelial cells
HUAEC	human umbilical artery endothelial cells
HUVEC	human umbilical vein endothelial cells
Ig	immunoglobulin
kdal	kilodalton
MAb	monoclonal antibody
mRNA	'messenger' ribonucleic acid

MTM	mixed thymocyte conditioned medium
NEAA	non-essential amino acids
OD	optical density
PA	plasminogen activator
PAP	peroxidase anti-peroxidase
PEG	polyethylene glycol
PF4	platelet factor 4
PMSF	phenylmethylsulphonylfluoride
PPACK	D-phenylalanyl-L-prolyl-L-arginine chloromethylketone
PR	progesterone receptor
PVDF	polyvinylidene difluoride
RGD(A)	arginine-glycine-asparagine-(alanine)
RIA	radioimmunoassay
RID	radial immunodiffusion
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFCM	serum-free conditioned medium
SFM	serum-free medium
SIBA	slot-immunobinding assay
TBS	Tris buffered saline
tPA	tissue plasminogen activator
Tris	Tris(hydroxymethane)aminomethane
TSP	thrombospondin
uPA	urokinase-type plasminogen activator
vWF	von Willebrand factor

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## CHAPTER ONE

### INTRODUCTION

#### 1.1 Discovery of thrombospondin

Baenziger et al (1971, 1972) first described a thrombin-sensitive protein of human platelets which was rapidly released upon stimulation of platelets with thrombin. This 190 kdal glycoprotein accounted for 20 - 30% of the total protein released.

The thrombin-sensitive protein was later characterised by Lawler et al (1977, 1978) under non-denaturing conditions. They named the intact 450 kdal glycoprotein thrombospondin (TSP) and suggested that each of the three constituent disulphide-linked 142 kD polypeptide chains were equivalent to the protein of Baenziger et al.

TSP was independently described by George (1978) as glycoprotein G, indicating its granule origin, and has also been given the names GP-160 or GP (McPherson et al 1981, Mosher et al 1982) and glycoprotein Ig (Nurden et al 1982).

Other workers demonstrated that TSP was located in platelet  $\alpha$ -granules (Hagen 1975, Gerrard et al 1980, Gogstad et al 1982, McLaren 1983, Wencel-Drake et al 1985) where it may account for 2 - 4% of the total platelet protein (Silverstein et al 1986).

## 1.2 Isolation of TSP

The original isolation method of Baenziger et al (1972) used a supernatant from thrombin-stimulated platelets. They separated the components by SDS-PAGE under reducing conditions and eluted the protein from the gel. The starting material has remained the same in all later methods although several other separation techniques have been employed.

Lawler et al (1978) used gel filtration on a Sepharose 4B column in the presence of EDTA. They found that TSP could be purified further by application of the gel-filtered protein onto a heparin-Sepharose 4B column and eluting stepwise with buffers containing 0.15, 0.25, 0.45 and 2.0 M NaCl (TSP eluted at 0.45 M NaCl). In later publications by this group, 2 mM  $\text{CaCl}_2$  was substituted for EDTA and the procedure was reversed, with heparin affinity chromatography becoming the first step (Lawler et al 1982, 1985).

Affinity chromatography on Lens culinaris lectin-Sepharose 4B was used by Dawes et al (1983), while Tuszynski et al (1985) employed a fibrinogen-Sepharose column to obtain highly purified TSP preparations. A recent method used a gelatin-Sepharose column coupled to a heparin-Sepharose column, both equilibrated in buffer containing 0.15 M NaCl, followed by elution of the heparin column only with increasing NaCl concentrations, and finally gel filtration of the TSP peak on a BioGel A-0.5m column (Santoro & Frazier 1987).

Other methods for isolating TSP which have been reported include barium chloride precipitation (Alexander & Detwiler 1984) and anion-exchange chromatography on a Mono-Q column using fast protein

liquid chromatography (Clezardin et al 1984).

### 1.3 Structure of TSP

The original description of the 190 kdal thrombin-sensitive protein (Baenziger et al 1972), containing 3.9% sialic acid and 1.6% N-acetylglucosamine, with 25% acidic and 25% nonpolar amino acids, was expanded and refined as more structural information became available with advancing techniques.

Lawler et al (1978) reported the amino acid and carbohydrate compositions of native TSP, noting that there was a relatively high proportion of aspartate/asparagine, glutamate/glutamine and cysteine residues and a relatively low proportion of leucine residues. The glycoprotein contained 0.7% sialic acid, 1.4% N-acetylglucosamine and 1.9% neutral sugars, had an isoelectric point of 4.7, and exhibited a heparin-binding activity. Their early electron microscope images showed a filamentous structure sometimes with nodules at the ends or occasionally along its length. Trypsin degradation resulted in a 210 kdal core fragment and three 70 kdal fragments.

More detailed measurements of the physical properties of TSP in solution containing EDTA were made by Margossian et al (1981). They concluded that the structure of TSP was largely stabilised by disulphide linkages, since they found a relatively low proportion of residues in ordered secondary structures yet the molecule did not unfold greatly in 6 M guanidine hydrochloride. The same group then compared the measurements they had made in EDTA solution with others made in the presence of  $\text{Ca}^{2+}$  (Lawler et al 1982) and showed that  $\text{Ca}^{2+}$  stabilised some parts of the TSP molecule. The physical

Table 1-1. Physical properties of TSP

	2mM Ca <sup>2+</sup>	1mM EDTA
Molecular weight (nonreduced)	no data	450000 <sup>a</sup>
	"	420000 <sup>b</sup>
(reduced)	"	185000 <sup>c</sup>
	"	145000 <sup>a</sup>
	"	133000 <sup>b</sup>
Sedimentation coefficient	9.7 S	8.6 S
Intrinsic viscosity	21 ml/g	40 ml/g
Axial ratio of prolate ellipsoid model	3.3	9.3
Electron microscopic length	54 nm	63 nm
Thrombin-generated fragments <sup>d</sup>	160000 <sup>c</sup>	160000 <sup>c</sup>
		135000 <sup>c</sup>
	125000 <sup>a</sup>	135000 <sup>a</sup>
		125000 <sup>a</sup>
		115000 <sup>a</sup>

<sup>a</sup> Weber-Osborn SDS-PAGE

<sup>b</sup> Sedimentation equilibrium

<sup>c</sup> Laemmli SDS-PAGE

<sup>d</sup> Principal high molecular weight fragments only



data (table 1-1) were in good agreement with higher quality electron microscope images, which were interpreted as a nodular rod-shaped molecule containing three polypeptide chains. When  $\text{Ca}^{2+}$  was removed, the nodular domains were thought to unravel to some extent, causing an increase in the length of the thin flexible connecting regions. Concomitantly, TSP became more susceptible to proteolysis by thrombin. The  $\text{Ca}^{2+}$ -sensitive nature of the TSP molecule was also demonstrated recently by ESR spin-label techniques (Slane et al 1988).

From further electron microscopy studies and the isolation of a 30 kdal heparin-binding peptide from the amino terminus of TSP, Coligan and Slayter (1984) proposed a crude model of the molecule. These studies were extended by Lawler et al (1985) and Galvin et al (1985). Both groups used information gained from proteolytic digestion of TSP, detection of the fragments with panels of monoclonal antibodies, and electron microscopy, to arrive at similar schematic models of the structural and functional organisation of the TSP molecule. They suggested that each polypeptide chain consisted of four distinct segments having specific binding activities for heparin, type V collagen, fibrinogen or platelets.

In 1986, Lawler and Hynes published the complete amino acid sequence of TSP. They used a cDNA library constructed from human endothelial cell TSP mRNA cloned in a bacteriophage, and determined the amino acid sequence from the nucleotide sequence of the coding region. Their results predicted a molecular weight of 127,524 (without carbohydrate) for each of the three chains of TSP (1152 amino acids long) and were consistent with all the previously

published structural and functional data on TSP. Three types of repeating sequence could be identified within the chains. Type 1 repeats (57 amino acids) showed homology with circumsporozoite proteins from *Plasmodium falciparum* and a cluster of type 1 repeats were found in a region of the molecule thought to bind several matrix proteins. Type 2 repeats (50 - 60 amino acids) had some homology with mouse epidermal growth factor (EGF) precursor and occurred in the central region of the molecule, next to the type 1 repeats. Other proteins, including growth factors, plasminogen activators and coagulation factors IX and X also have a region of EGF-like homology (Bender 1985) and some, like TSP, have binding activities with plasminogen, fibronectin and fibrinogen (see section 1.4). Type 3 repeats (38 amino acids) showed homology with the calcium-binding sites of parvalbumin and calmodulin. A group of eight type 3 repeats was found in that part of the molecule previously thought to be sensitive to  $\text{Ca}^{2+}$ ; thus they may constitute a series of adjacent  $\text{Ca}^{2+}$ -binding sites in TSP. The last of the type 3 repeats was also found to include the sequence arg-gly-asg-ala (RGDA) and may represent the site for the association of TSP with cells (Ruoslahti & Pierschbacher 1986), especially platelets.

Other studies by Clezardin et al (1986a,b) have identified subtle differences between platelet TSP and endothelial and fibroblast TSP. We used a panel of monoclonal antibodies and a polyclonal antibody (section 2.3) to detect trypsin- and thermolysin-generated fragments, separated by fast protein liquid chromatography using a Superose 6 or 12 gel filtration column. It was also observed that TSP oligomers from the cell cultures were

more resistant to proteolysis than oligomers of platelet TSP. Our findings were supported by the data of Dardik and Lahav (1987).

By combining the information from primary sequence data, electron microscopic shape and protein biochemistry studies, Lawler and Hynes (1986) produced a more comprehensive model of the structural and functional organisation of the TSP molecule (figure 1-1).

Most recent work, leading on from the amino acid sequence of Lawler and Hynes (1986), has identified several homologies between sequences in TSP and others found in the following proteins; von Willebrand factor, procollagen, thrombomodulin, vitronectin, plasma cell membrane glycoprotein PC-1, complement components C9, C8 $\alpha$ , C8 $\beta$  and C7, properdin, and proteins isolated from the malaria parasite *Plasmodium falciparum* (Hunt & Barker 1987, Patthy 1988, Robson et al 1988, Goundis & Reid 1988).

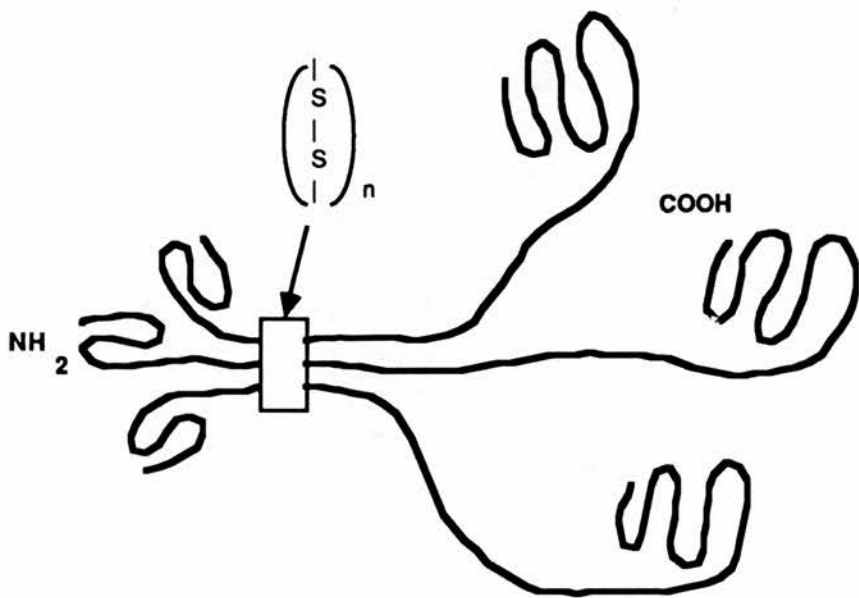
Studies of the TSP gene and messenger sequences (Donoviel et al 1988, Hennessy et al 1989) have already given an insight into its evolutionary ancestry and the regulatory mechanisms which may operate at the level of TSP synthesis within cells. Further studies of this kind, together with those aimed at defining the precise biochemical interactions of the molecule, will greatly increase the understanding of the structure and function of TSP.

#### 1.4 Interactions of TSP with other macromolecules

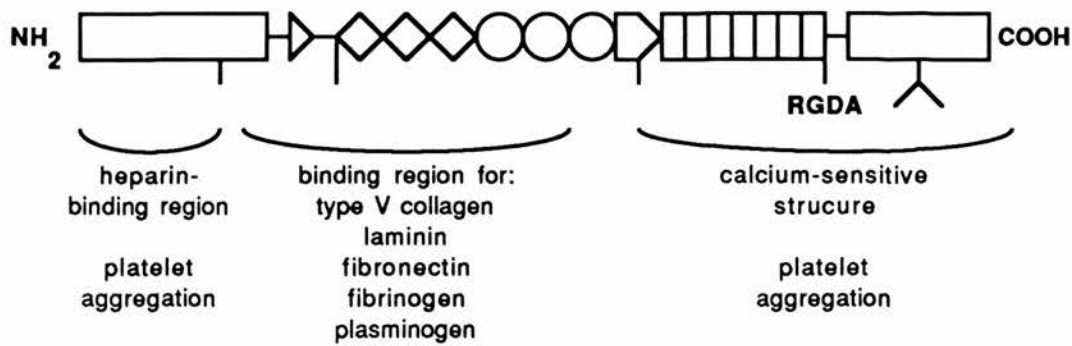
TSP is known to bind to a wide range of macromolecules involved in the formation of extracellular matrices and in the regulation of thrombosis and haemostasis (the fibrin clot may also be considered as a specialised form of extracellular matrix). It has therefore

**Fig. 1-1. Schematic model of the structural and functional organisation of the TSP molecule**

(From Lawler & Hynes 1986)



**Domain structure of the TSP monomer:**



been termed a multifunctional glycoprotein (Silverstein et al 1986, Lawler 1986, Asch & Nachman 1989).

#### 1.4.1 Heparin

The interaction of TSP with heparin and related mucopolysaccharides has been well characterised and is commonly utilised in procedures for the purification of TSP (sections 1.2 and 2.1.2). Low molecular weight heparin binds to the 25-30 kdal amino-terminal domain of TSP with high affinity (Dixit et al 1984a, Raugi et al 1984, Dardik & Lahav 1987). Binding was reduced at high ionic strength and inhibited by competition with other mucopolysaccharides and platelet factor 4 (Slayter et al 1987).

The biological significance of the TSP-heparin interaction is not known, but it has been proposed that the cell surface receptor for TSP on fibroblasts and endothelial cells may be a heparin-like molecule (Murphy-Ullrich & Mosher 1987b). Also, heparin and other highly charged polymers are able to inhibit the complement system via component C1q (Ecker & Pillemer 1941, Confrancesco et al 1979, Silvestri et al 1981). It is possible that such interactions may be important during wound healing and developmental processes, for example at sites of vascular injury and in atherosclerosis.

#### 1.4.2 Fibrinogen

Leung and Nachman (1982) observed the formation of a complex between TSP and fibrinogen which was inhibited by certain amino sugars and which was independent of the presence of calcium. There is, however, some disagreement over the effect of calcium ions on the TSP-fibrinogen interaction (Lawler 1986). The region of the TSP

molecule which contains the fibrinogen-binding site has been located to a 40 kdal fragment near the carboxy-terminus (Dixit et al 1984b). Binding of TSP could be inhibited by antibodies directed against the carboxy-terminus of the  $\alpha$ -chain of fibrinogen (Tuszynski et al 1985) whilst Bacon-Baguley et al (1987) showed that TSP bound to both the A $\alpha$  and B $\beta$  chains but not the  $\gamma$ -chain of reduced fibrinogen.

#### 1.4.3 Fibronectin and collagen

Although a specific interaction between TSP and fibronectin has been observed (Lahav et al 1982), the properties of the binding site are not well characterised (Leung & Nachman 1982, Lahav et al 1984). Walz et al (1987) reported that TSP bound to both chains of reduced fibronectin but noted that progress had been hampered by technical difficulties in handling fibronectin.

TSP was found to bind to type V collagen with high affinity (Mumby et al 1984). Galvin et al (1987) confirmed this observation and defined the high affinity binding site. Their studies also revealed a low affinity site common to collagen types I-V. The collagen-binding site of TSP has been mapped to a 70 kdal chymotryptic fragment which represents the thin flexible connecting region of the molecule (Galvin et al 1985).

Since fibronectin and collagen are major components of the extracellular matrix, it is likely that interactions between TSP and these macromolecules are important for the stability and regulation of the matrix. Indeed, TSP has been localised by immunocytochemical techniques in the extracellular matrix of certain cell types where it stains in a fibrillar pattern similar

to that of fibronectin (Raugi et al 1982, Jaffe et al 1983).

It is also of interest that the 70 kdal fragment of TSP binds laminin, a component of basement membranes (Liotta et al 1986), in an ELISA (Mumby et al 1984) although this has not been confirmed using fluid-phase techniques.

#### 1.4.4 Plasminogen and histidine-rich glycoprotein

The interactions between TSP, plasminogen, tissue-type plasminogen activator (tPA) and histidine-rich glycoprotein (HRGP) have been studied in detail by Silverstein and coworkers (reviewed in Silverstein et al 1986). They have demonstrated the formation of complexes between TSP, plasminogen and HRGP, and between TSP, plasminogen and tPA (Silverstein et al 1985a,b).

HRGP has been shown to interact with the lysine-binding site of plasminogen (Lijnen et al 1980) and may therefore inhibit fibrinolysis by reducing the binding of plasminogen to fibrin. HRGP also binds to heparin and neutralises its anticoagulant properties (Lijnen et al 1984). Thus local binding of TSP to plasminogen and HRGP at or near the platelet surface may regulate fibrinolysis and coagulation.

Plasminogen activation by tPA is unfavourable in the absence of a fibrin substrate and proceeds with optimal efficiency on the clot surface (Hoylaerts et al 1982). However, activation was found to occur at an intermediate rate on a TSP surface (Silverstein et al 1985b) and therefore interactions between TSP, plasminogen and tPA may be important in extracellular proteolytic events, for example in cell migration, tissue breakdown and repair.

## 1.5 Distribution of TSP in cells and tissues

Initially TSP was thought to be a platelet-specific protein like platelet factor 4 (PF4) and  $\beta$ -thromboglobulin (BTG) which also occur in platelet  $\alpha$ -granules. Results of immunocytochemical staining by McLaren (1983) suggested that platelet TSP was synthesised in megakaryocytes, and this view was confirmed by the  $^{35}\text{S}$ -methionine protein-labelling studies of Rabellino et al (1985) using cultured megakaryocytes.

The first report of TSP synthesis by cultured endothelial cells (McPherson et al 1981) was followed by several others for endothelial cells derived from various human sources (Mosher et al 1982, Hunter et al 1984, Kramer et al 1985). Proteins similar to TSP, but not identified as such, had earlier been shown to be synthesised by hamster embryo fibroblasts (Carter & Hakomori 1978) and HT-1080 sarcoma cells (Alitalo et al 1980). Since then, a wide variety of cell types have been shown to produce TSP in vitro (table 1-2).

However, it has proved more difficult to demonstrate TSP in tissues in vivo. Using affinity purified rabbit antibody to human TSP, Wight et al (1985) localised TSP in the extracellular matrices of some human tissues and specifically in the basement membranes of others. Thus, TSP was present in the peritubular connective tissue of kidney, at the base of glandular epithelia within skin and lung, at the dermal-epidermal junction in skin, and in the interstitial connective tissue of skeletal muscle. It was also observed around small blood vessels and along the luminal surface of aorta; areas of vessels characterised by atherosclerosis exhibited marked immunostaining for TSP.



Table 1-2. Occurrence of TSP in cultured cells

Cell type	Reference
HT-1080 sarcoma	Alitalo et al 1980
Endothelial (bovine aortic)	McPherson et al 1981
(human umbilical vein)	Mosher et al 1982
(human, various)	Hunter et al 1984
(human microvascular)	Kramer et al 1985
Smooth muscle	Raugi et al 1982
Fibroblast (hamster embryo)	Carter & Hakomori 1978
(human foreskin)	Raugi et al 1982
(human, various)	Jaffe et al 1983
Type II alveolar epithelial	Sage et al 1983
Monocyte	Schwartz et al 1983
Macrophage	Jaffe et al 1985
Megakaryocyte	Rabellino et al 1985
Glial (human brain)	Asch et al 1986
Glomerular mesangial	Raugi & Lovett 1987
Keratinocyte	Wikner et al 1987
Articular chondrocyte	Miller & McDevitt 1988
Avian thrombocyte	Wachowicz et al 1988
Melanocyte	McClenic et al 1989
Osteoblast	Robey et al 1989
MCF-7 human breast cancer	Pratt et al 1989

In contrast, Murphy-Ullrich and Mosher (1987a) failed to obtain specific staining of various adult human tissues with monoclonal or polyclonal antibodies to human platelet TSP, but did observe extensive staining of loose connective tissue in sections of human foetal limb. They suggested that TSP may only be a major matrix component during a specific period of an organ's development. Furthermore, O'Shea and Dixit (1988) observed a unique distribution of TSP in the extracellular matrix of the developing mouse embryo.

TSP has also been shown to be associated with wound tissue (Raugi et al 1987) and thrombus formation (Murphy-Ullrich & Mosher 1985, Perlman et al 1987). Recent reports have identified TSP in cartilage (Miller & McDevitt 1988) and bone (Robey et al 1989).

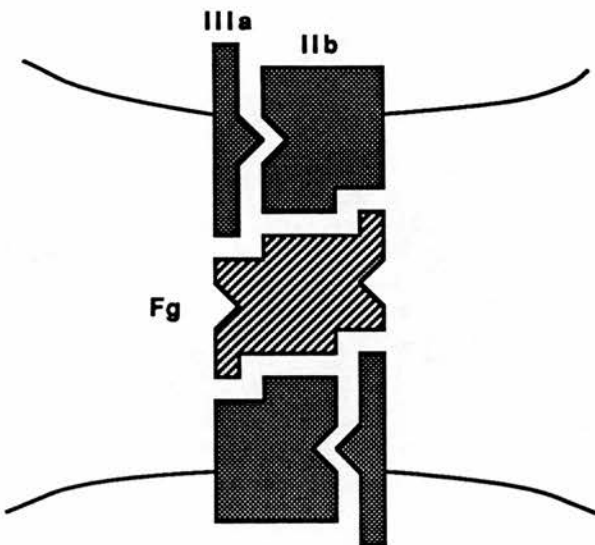
#### **1.6 TSP and platelet function**

Platelets play an essential role in haemostasis. Following vascular injury, platelets adhere to the subendothelial matrix and become activated, releasing the contents of their  $\alpha$ -granules. The stimulated platelets aggregate, more platelets are recruited and eventually form a haemostatic plug (thrombus). The aggregate is probably stabilised by interactions between the platelet membrane glycoprotein IIb/IIIa complex (GpIIb/IIIa), fibrinogen, TSP and possibly other secreted glycoproteins, fibronectin and von Willebrand factor (vWF). This model (fig.1-2) is supported by the work of Rybak (1986) who found that GpIIb/IIIa and TSP incorporated into liposomes formed aggregates in the presence of  $\text{Ca}^{2+}$  and fibrinogen, and could also bind fibronectin and vWF.

Platelet aggregation can be described as a two-step process. In the primary phase, stimulation with various physiological agents

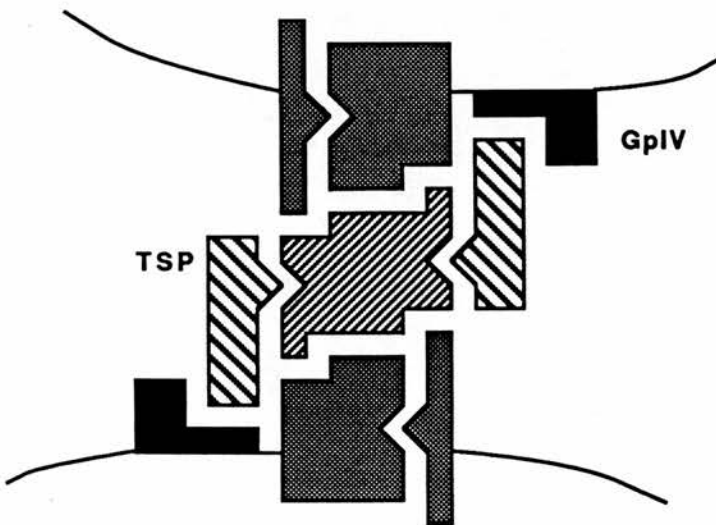
**Fig. 1-2. Molecular model of platelet aggregation**

(From Asch & Nachman 1989)



**Primary phase:**

Platelet activation causes clustering of GpIIb/IIIa complex on platelet membrane surface and binding of fibrinogen (Fg)



**Secondary phase:**

Secretion of alpha-granule contents causes binding of TSP to the receptor GpIV and to fibrinogen, with stabilisation of the platelet-platelet interaction

(e.g. thrombin, collagen, ADP and adrenaline) causes a change in platelet shape, clustering of the GpIIb/IIIa complex on the membrane surface and binding of fibrinogen (Mustard et al 1978, Bennett & Vilaire 1978, George et al 1980, Nachman and Leung 1982). The secondary, secretion-dependent phase, is probably mediated by the major  $\alpha$ -granule components, resulting in the irreversible formation of platelet aggregates (Plow et al 1984, George et al 1984).

Three main pieces of evidence support a role for TSP in the aggregation of platelets; i) TSP can become associated with the platelet surface, by a calcium-dependent mechanism involving fibrinogen and GPIIb/IIIa, and by a calcium-independent mechanism involving an, as yet, unidentified receptor (George 1978, Phillips et al 1980, Gartner and Dockter 1984, Asch et al 1985, Hourdille et al 1985, Plow et al 1985, Wolff et al 1986, Aiken et al 1987); ii) TSP is thought to be the endogenous platelet lectin, responsible for the ability of stimulated platelets to agglutinate fixed red blood cells (reviewed by Gartner 1987); iii) anti-TSP antibodies can inhibit platelet aggregation (Nurden et al 1983, Agam et al 1984, Gartner et al 1984, Leung 1984, Dixit et al 1985).

Although the specific role of TSP in platelet function remains unclear, it would seem that multiple sites on the TSP molecule are involved in binding to the platelet membrane and to fibrinogen, resulting in the transformation of the initial microaggregate into an irreversible macroaggregate (Silverstein et al 1986).

## 1.7 Clinical disease and TSP in the circulation

In normal individuals, TSP is present in plasma at low concentrations detectable by radioimmunoassay (Saglio & Slayter 1982, Dawes et al 1985, Switalska et al 1985) and rises over 100-fold in serum due to its release from platelets during the clotting process. Two clinical syndromes are known in which defects in platelet TSP have been identified.

Grey platelet syndrome (Raccuglia 1971) is a rare inherited bleeding disorder characterised by a lack of platelet  $\alpha$ -granules and, hence, a marked deficiency of all  $\alpha$ -granule proteins including TSP (Nurden et al 1982). Platelets from patients with this disorder are often large agranular cells with a distinctive grey colour, but have a normal content of mitochondria, dense bodies, peroxisomes and lysosomes (White 1979, Breton-Gorius et al 1981, Berndt et al 1983). Grey platelets have been shown to give decreased aggregation in response to ADP, thrombin and collagen (Gerrard et al 1980) and to lack the surface-bound haemagglutination activity associated with activated platelets which is mediated by TSP (Gartner et al 1981).

Essential thrombocythemia, one of a group of myeloproliferative disorders, is characterised by elevated platelet/megakaryocyte elements in the circulation and frequent bleeding and thrombotic complications (Schafer 1984). Impaired platelet function is commonly observed in these patients (Spaet et al 1969) and is associated with a variety of molecular defects (Bolin et al 1977, Keenan et al 1977, Cooper et al 1978, Castaldi et al 1982, Schafer 1982). Platelet TSP has been shown to be more susceptible to proteolysis in essential thrombocythemia (Booth et al 1984, Lawler

et al 1986) and this may be due to its defective glycosylation (Clezardin et al 1985).

Most interest in the measurement of circulating TSP levels has centred on its use as a marker of platelet activation. In general, increases in plasma TSP correlate with increases in the levels of the specific platelet  $\alpha$ -granule components BTG and PF4. Although BTG is generally the most sensitive marker of in vivo platelet activation (Lane et al 1984, Ffrench et al 1985), measurement of plasma TSP has been found to be useful in patients whose disease is complicated by impaired renal function (Trzeciak et al 1985) and in patients with vasculitis (McCrohan et al 1987). Several factors probably explain why TSP is not always useful for monitoring in vivo platelet activation. TSP is synthesised and secreted by a variety of cells other than platelets, including those of the endothelium, which may make a significant contribution to the total plasma concentration (Dawes et al 1988). TSP may bind to the platelet surface and to several plasma proteins, and is incorporated into fibrin clots; mechanisms which would remove TSP from plasma following its release from activated platelets (Booth & Berndt 1987).

#### **1.8 Relationship of TSP to malignant disease**

During metastasis, tumour cells detach from the primary cancer, penetrate basement membrane and endothelium, disseminate through the circulation (avoiding host defence mechanisms), adhere to and migrate through the endothelium again, and finally invade the interstitial tissue to establish a secondary cancer (Poste & Fidler 1980). It has long been recognised that platelets play an important

role in tumour cell metastasis (Gasic et al 1968), while several lines of evidence implicate TSP in the development of malignant disease (Asch & Nachman 1989).

Specific receptor sites for TSP have been identified on the surface of platelets, endothelial cells and a variety of human tumour cells (Asch et al 1987). The binding of TSP to components of the extracellular matrix and basement membrane has already been discussed (section 1.4.3); TSP could therefore be involved at several points in the metastatic process through potential interactions with extracellular matrix, endothelium or circulating platelets.

TSP promotes the attachment and spreading of several human tumour cells in vitro (Varani et al 1986, Roberts et al 1987, Tuszynski et al 1987b) and a recent report showed that TSP potentiates tumour cell metastasis in an animal model (Tuszynski et al 1987a).

Tumour cells are known to secrete a variety of substances which directly or indirectly may degrade components of the extracellular matrix and basement membrane (Liotta et al 1979, Kramer et al 1982, Laug et al 1983, Yee & Shiu 1986). In particular, plasmin(ogen) could potentially be localised at the tumour cell surface via binding to TSP (section 1.4.4), where its proteolytic activity might facilitate migration of the tumour cell through extracellular matrix and basement membrane.

## 1.9 TSP and the human breast

### 1.9.1 Breast structure and development

The structure and development of the mammary gland has been reviewed by Cowie (1974), Vorherr (1974) and Russo et al (1982).

The human breast is composed of glandular (parenchymal) tissue, comprising lobules of alveoli (or acini) drained by a tree-like duct system which opens at the nipple, embedded in supporting tissue, which consists mainly of connective (stromal) tissue, together with adipose tissue. The breast is supported on the chest wall by a series of ligaments connecting the stroma with the pectoral muscles, and by sheets of connective tissue (fascia) which envelop the gland. The blood and nerve supply to the breast is similar to that of the surrounding skin and this is consistent with the cutaneous origin of the gland. However, the nipple and areolar region are highly innervated with sensory nerve fibres, whereas glandular structures within the breast are probably supplied by a sympathetic nerve system only.

The breast originates from the ectoderm of the foetus. This thickens to form the milk-lines, from which buds proliferate and invade the surrounding mesenchyme to constitute a primordial duct system. The development of the breast involves continuous changes influenced by body growth from birth through to senility, with superimposed effects due to the rhythmical hormonal stimulation of the menstrual cycle. As a result, the gland becomes a complex and extremely heterogeneous histological structure, due to unequal responses of the many lobules to their local hormonal environments.

Full differentiation of the acini into milk-secreting units only



occurs during pregnancy and lactation, when progressive hypertrophy of the breast takes place. Even then, differentiation of acini occurs unevenly throughout the gland, and through whole lobules. The secretory acinus is lined by a single layer of cuboidal epithelial cells which synthesise milk fat, protein and carbohydrate, and is bound by a thin basement membrane in close contact with capillaries in the surrounding tissue. It has been suggested that the least differentiated lobules may be the most likely targets for neoplastic transformation by carcinogens (Russo et al 1982).

#### **1.9.2 Breast secretions**

Analysis of breast secretions, either spontaneous or collected by nipple aspiration, has been used as a convenient and non-invasive method for investigating breast function and disease (Petrakis 1986, Dairkee & Hackett 1986). It is thought that the non-lactating breast secretes and reabsorbs a wide variety of substances, some of which may be retained, concentrated and metabolised by breast epithelium and possibly other cells in breast fluid such as foam cells and macrophages. Cytological analysis of nipple aspirates may be useful in the diagnosis of breast disease; thus a close correlation between occurrence of abnormal epithelial cell types in breast fluid and proliferative disease in corresponding breast tissue has been shown (Sartorius et al 1977, King et al 1983).

Colostrum and milk, the secretions produced by the lactating breast during late pregnancy and after parturition respectively, contain a wide range of electrolytes, carbohydrates and proteins

(in addition to fat globules and cellular elements). The concentration of these components change considerably around the time of parturition (Bezkorovainy 1977, Kulski & Hartmann 1981).

TSP was previously measured in human colostrum and milk, other breast secretions, and in goat colostrum and bovine milk (Dawes et al 1985, 1987). Breast secretions contained high concentrations of TSP (range = 1 - 145 ug/ml, median = 23 ug/ml) relative to those in plasma, as did milk and colostrum. In the goat, TSP levels fell sharply during the transition from colostrum to milk. TSP isolated from this source showed a similar affinity for heparin-Sepharose as did human platelet TSP, and a similar molecular weight of 450 kdal by gel filtration chromatography. However, the colostrum protein contained more fragments of low molecular weight and a different fragmentation pattern after thermolysin digestion, indicating differences in amino acid sequence between TSP isolated from goat colostrum and other sources (Dawes et al 1987).

### 1.9.3 Cyst fluids

Breast cysts are a common benign lesion of Western women, and those with cystic disease may have an increased risk of developing breast cancer (Haagensen et al 1981). Several studies have been done on the composition of cyst fluid and have measured proteins (Yap et al 1984, Miller & Dawes 1985), hormones (Raju et al 1977, Bradlow et al 1981a, Miller et al 1982), electrolytes (Gatzky et al 1979, Bradlow et al 1981b) and cells (Cowen & Benson 1979). Despite this, the mechanism by which cyst fluid is formed is not known.

Two types of breast cyst have been identified which differ in the nature of the epithelial lining (Dixon et al 1983) and the

composition of the cyst fluid (Miller et al 1983a); group I cysts have an apocrine epithelium and high concentrations of  $K^+$  and DHA-sulphate; group II cysts have a flattened epithelium and high concentrations of  $Na^+$ . The levels of TSP in these two types of cyst fluid are also different, being significantly higher in group II ( $Na^+$ ) cysts (Miller & Dawes 1985). Since TSP concentrations were low in group I ( $K^+$ ) cysts, which have an active apocrine lining, this would suggest that apocrine epithelial cells are not the source of the high TSP levels observed in some cyst fluids.

#### 1.9.4 Breast cancer

Breast cancer is the most common form of malignancy in women world-wide, and accounted for 18% of all new cases of female cancer in 1980 (Parkin et al 1988). In the United States, where the incidence of the disease is amongst the highest, 1 in 14 women are expected to develop breast cancer sometime during their lives (Kelsey 1979). In general, the rates of breast cancer occurrence are low in Eastern and developing countries compared with the more affluent populations of Europe and North America (Parkin et al 1988). The disease is relatively uncommon in young women, but increases in frequency up to the menopause and more slowly thereafter (De Waard 1983, Boyle & Robertson 1987). In a study of 1000 cases of invasive breast cancer, Fisher et al (1975) described the main histological types of tumour (table 1-3). They found that infiltrating ductal carcinoma was present in over 80% of cases, either alone or in combination with other lesions.

A variety of genetic, environmental and hormonal factors appear to be associated with an increased risk of developing breast cancer

Table 1-3. Incidence of histological types of breast cancer

	%
Infiltrating ductal (pure)	52.6
Infiltrating ductal with tubular	16.5
Infiltrating ductal with other types	11.5
Medullary	6.2
Lobular invasive	4.9
Mucinous	2.4
Paget's disease	2.3
Combinations of types not including infiltrating ductal	1.6
Tubular	1.2
Adenocystic	0.4
Papillary	0.3
Carcinosarcoma	0.1
	<hr/> 100.0 <hr/>

From Fisher et al 1975

(table 1-4). The involvement of hormones, particularly the ovarian steroids (oestrogens), in the development of the normal and malignant breast is well established. However, only about one third of breast cancers respond to oestrogen deprivation therapy (Henderson & Canellos 1980), such as oophorectomy in premenopausal women, or administration of drugs which inhibit oestrogen biosynthesis (e.g. aminoglutethimide) or block oestrogen action (e.g. tamoxifen) (Miller 1987).

The responsiveness of breast tumour cells to oestrogens is thought to depend upon the presence of specific intracellular receptor proteins (Edelman 1975, Lippman & Allegra 1978). Thus, tumours containing measurable amounts of oestrogen receptors (ER) have a 50% chance of responding to endocrine therapy while ER negative tumours generally show response rates of less than 10% (Hawkins 1985). Presence of both ER and progesterone receptor (PR) may give an even better prognosis (Leclercq et al 1983) and probably indicates that the tumour has maintained its sensitivity to oestrogen, since PR synthesis is itself induced by oestrogen (Horwitz & McGuire 1978).

Table 1-4. Factors associated with increased risk of breast cancer

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Genetic	Family history of breast cancer
Environmental	Domicile Western Europe and North America Obesity and high fat and protein diet Irradiation ? viruses ? chemical carcinogens
Hormonal	Long menstrual life (early menarche, late menopause) Anovulatory menstrual cycles Nulliparity Late age at first pregnancy Prolonged high dose oestrogen therapy
Disease	Previous history of benign breast disease (epithelial hyperplasia)

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Modified from Vorherr & Messer 1978

## CHAPTER TWO

### MATERIALS AND METHODS

Unless stated specifically, chemicals and solvents were generally obtained from Sigma Chemical Co. Ltd., Poole, Dorset, BDH Chemicals Ltd., Poole, Dorset, or Fisons plc, Loughborough, Leics.

#### 2.1 Isolation of TSP from human platelets

Unless stated otherwise, the TSP used in assays and experiments described later was isolated from fresh human platelets after stimulation by thrombin, followed by affinity chromatography, ion-exchange chromatography and gel filtration chromatography.

##### 2.1.1 Preparation of a supernatant from thrombin-stimulated platelets (Baenziger et al 1972)

pH 6.5 buffer;

102 mM NaCl

22 mM  $\text{NaH}_2\text{PO}_4$

3.9 mM  $\text{Na}_2\text{HPO}_4$

3.9 mM  $\text{KH}_2\text{PO}_4$

5.5 mM glucose

pH 7.6 buffer;

15 mM Tris-HCl

140 mM NaCl

5 mM glucose

Freshly prepared platelet concentrates from 4 - 6 units of human blood were obtained from the Scottish National Blood Transfusion Service, Edinburgh and used within 1 h.

Platelet concentrates were pooled into plastic 250 ml bottles and centrifuged at 100 g (750 rpm) in a Sorvall RC-5B centrifuge with type HS-4 rotor (Du Pont Instruments, Stevenage, Herts) for 15 min to remove red cells. Platelet-rich plasma was decanted into universal containers and centrifuged at 600 g (2000 rpm; Sorvall RC-5B/HS-4 rotor) for 15 min to pellet platelets. Platelet-poor plasma was removed and the pellets resuspended in pH 6.5 buffer and centrifuged again. Washed platelets were resuspended in a suitable volume of pH 7.6 buffer in 50 ml flat-bottomed plastic containers and incubated for 5 - 10 min at 37°C. In most preparations the volume of pH 7.6 buffer used was 30 ml (2 x 15 ml) but it was preferable to adjust the volume to give approximately  $2 \times 10^9$  cells/ml since too high a platelet count may result in subsequent loss of TSP through precipitation from concentrated solution.

Human thrombin (Sigma product no. T6759) was added at 1 NIH unit/ml with gentle stirring, and after 2 min further stimulation was inhibited by the addition of 40 uM leupeptin, 10 uM D-phenylalanyl- L-prolyl-L-arginine chloromethyl ketone (PPACK; Calbiochem, Cambridge Bioscience, Cambridge) and 1 mM phenylmethylsulphonyl fluoride (PMSF). Ethylenediaminetetra-acetic



acid (EDTA) was added at a final concentration of 5 mM to dissociate platelet-bound TSP, and the aggregated platelets were allowed to settle at 0°C.

The supernatant from thrombin stimulated platelets was decanted into plastic tubes and centrifuged at 40000 g (18000 rpm; Sorvall RC-5B/SS-34 rotor) for 60 - 120 min at 4°C to remove cells and particulate material. The resulting supernatant was decanted into a clean plastic tube and processed further without delay.

#### **2.1.2 Affinity chromatography of thrombin-stimulated platelet supernatant on heparin-Sepharose CL-6B (Lawler et al 1978)**

A glass column (2 x 24 cm) was packed with 10 g heparin-Sepharose CL-6B freeze-dried powder (Pharmacia Ltd., Milton Keynes, Bucks) which had been pre-swollen and washed according to the manufacturer's instructions and equilibrated in 15 mM Tris-HCl, 0.15 M NaCl, 0.02% NaN<sub>3</sub>, pH 7.4.

Supernatant from thrombin-stimulated platelets was applied to the column and eluted at a flow rate of 0.4 ml/min with Tris-HCl buffer containing successively higher NaCl concentrations (0.15 M, 0.25 M, 0.55 M and 2.0 M). Fractions of volume 4 ml, collected during sample application and elution, were assayed for protein by a dye-binding method (see section 2.2). The TSP peak eluting at 0.55 M NaCl was stored at 4°C in the presence of 1 mM PMSF. In some experiments this material was purified further by anion-exchange chromatography and gel filtration chromatography.

### 2.1.3 Purification of TSP by anion-exchange chromatography on a Mono-Q column (Clezardin et al 1984)

The TSP peak from the heparin column was concentrated in an ultrafiltration cell with a PM10 membrane (Amicon Ltd., Woking, Surrey) and diluted 1:3 - 1:4 in start buffer (20 mM triethanolamine, pH 7.4). This solution was applied, via a 50 ml Superloop, to a Mono-Q HR 5/5 anion-exchange column connected to a Fast Protein Liquid Chromatography (FPLC) system (all from Pharmacia) at a flow rate of 0.5 ml/min. The column was washed with start buffer until the absorbance of the eluate at 280 nm returned to zero, then eluted with a 0 - 1 M NaCl gradient generated over 30 min. The final buffer was 20 mM triethanolamine, 1 M NaCl, pH 7.4. Fractions containing the TSP peak, identified by radioimmunoassay, were pooled and concentrated by ultrafiltration as before.

### 2.1.4 Purification of TSP by gel filtration on a Superose 6 column (Clezardin et al 1986a)

The TSP solution was injected (500 ul, flow rate 0.5 ml/min) onto a Superose 6 HR 10/30 gel filtration column (Pharmacia) connected to an FPLC system and eluted with 20 mM triethanolamine, 0.35 M NaCl, pH 7.4 buffer. Fractions containing the TSP peak, identified by radioimmunoassay, were pooled and stored at 4°C in the presence of 0.02% w/v  $\text{NaN}_3$ .

## 2.2 Protein assay

Samples were assayed for total protein by the Bio-Rad protein assay, based on the method of Bradford (1976). In some experiments the assay was performed according to the manufacturer's instructions (Bio-Rad Laboratories Ltd., Watford, Herts) but in others, especially where many samples were involved, a modified procedure was used as follows:

Samples (50  $\mu$ l) and diluted (20% v/v) dye reagent (2.5 ml) were mixed in plastic tubes. After incubation at 20°C for 15 - 60 min, aliquots (200  $\mu$ l) were transferred to the wells of a 96-flat bottom well microtitre plate (Sterilin Ltd., Feltham, Middlesex). The absorbance of each well at 620 nm was measured in a Titertek Multiskan MCC/340 photometer (Flow Laboratories Ltd., Irvine, Ayrshire) against a reagent blank. For each assay, a standard curve was constructed using bovine serum albumin (BSA) solutions from 0.2 - 1.4 mg/ml.

## 2.3 TSP radioimmunoassay

The method used was that of Dawes et al (1983) with minor modifications.

### 2.3.1 Iodination of TSP

TSP isolated from human platelets was iodinated in early experiments by the chloramine T method and in later experiments by an Iodogen coated tube method. Iodination using Iodogen was advantageous in that (i) a more dilute solution of TSP could be used, (ii) tracer of lower specific radioactivity could be produced which had better stability and retention of binding activities, and

(iii) the procedure was simpler to perform.

#### 2.3.1.1 Chloramine T method (Greenwood et al 1963)

The following reagents were dispensed into a plastic tube as separate drops;

5 ul  $^{125}\text{I}$  (100 mCi/ml,  $\text{I}^-$  in NaOH; Amersham International plc, Amersham, Bucks)

5-20 ul antigen (at least 500 ug/ml in aqueous solution)

10 ul chloramine T (5 mg/ml in 0.25 M phosphate buffer, pH 7.5)

The tube was mixed manually for 5 sec and the reaction stopped by the addition of 0.86 ml  $\text{Na}_2\text{S}_2\text{O}_5$  (160 ug/ml in 0.05 M phosphate buffer, pH 7.5) and 100 ul KI (10 mg/ml in 0.05 M phosphate buffer, pH 7.5). Protein was separated from unreacted  $^{125}\text{I}$  by gel filtration on a 1 x 10 cm glass column packed with Sephadex G-50 Fine (Pharmacia), equilibrated and eluted with 0.05 M phosphate buffer, 2.0% horse serum (Wellcome Diagnostics, Dartford), 1.0% Tween 20, pH 7.5. A specific activity of about 1100 KBq/ug was routinely obtained.

#### 2.3.1.2 Iodogen coated tube method (Salacinski et al 1981)

Plastic micro-reaction tubes were coated internally with 20 ug Iodogen (1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycouril; Pierce and Warriner (UK) Ltd., Chester, Cheshire) by evaporation under nitrogen from a methylene chloride solution (200 ug/ml). The Iodogen coated tubes were stable for several months at -40°C. TSP (5 - 20 ug) in aqueous solution was mixed in the tube with buffer

(0.05 M phosphate buffer, 0.01% Tween 20, pH 7.5) up to a total volume of 100  $\mu$ l, and 1 - 5  $\mu$ l  $^{125}\text{I}$  (100 mCi/ml,  $\text{I}^-$  in NaOH; Amersham). After 5 min the reaction was terminated by transfer of the solution to a clean plastic tube. After a further 10 min, protein was separated from unreacted  $^{125}\text{I}$  by gel filtration as before. Specific activities of 100 - 2000 KBq/ $\mu$ g were obtained, depending on the ratio of TSP to  $^{125}\text{I}$  used.

### 2.3.2 Production of primary antibody

Polyclonal rabbit anti-TSP was provided by Dr G.O. Gogstad, Research Institute for Internal Medicine, University of Oslo, Norway and had been produced as follows. TSP from human platelets stimulated with polystyrene latex particles (Dow Chemical Co., Midland, USA) and purified by gel filtration on a Sephacryl S-300 column followed by affinity chromatography on a Lens culinaris lectin-Sepharose 4B column, was used as immunogen. A 1:1 v/v solution of TSP and Freund's incomplete adjuvant (Gibco Ltd., Paisley, Renfrewshire) was injected subcutaneously into rabbits. Four injections of 60  $\mu$ g TSP/rabbit were given at 2-weekly intervals, followed by 30  $\mu$ g at 4-weekly intervals. Blood was collected 10 and 20 days after each of these injections. The pooled antiserum from 3 rabbits was adsorbed with platelet membranes. It was monospecific for TSP and did not crossreact with BTG, PF4, fibrinogen, fibronectin or vWF.

### 2.3.3 Preparation of solid-phase secondary antibody

Donkey anti-rabbit IgG (Scottish Antibody Production Unit, Carlisle, Lanarkshire) was coupled to Sepharose 4B (Pharmacia) by the method of Hunter and Budd (1981). Briefly, 80 g pre-swollen Sepharose 4B was reacted with 8 g CNBr at pH 10 - 11. After extensive washing with 0.1 M NaHCO<sub>3</sub> followed by 0.1 M citric acid, pH 6, the activated gel was mixed with 20 ml antiserum by inversion overnight at 20°C. Glycine (final concentration 0.2 M) was added and mixed a further 2 h. The coupled gel was washed extensively with 0.1 M citric acid, pH 6 followed by 50 mM phosphate buffer, pH 7.5 and 50 mM phosphate buffer, 1% Tween 20, pH 7.5. The product was stored at 4°C as a 1:1 v/v solution in the final wash buffer. The working dilution in the assay was optimised for each batch but was usually 1:5 v/v.

### 2.3.4 Assay procedure

The assay was carried out in 50 mM sodium phosphate, 2% horse serum (Wellcome), 1% Tween 20, pH 7.5. Duplicate plastic tubes were set up for each test containing 50 ul standard or sample, 50 ul primary antibody (1/800 dilution) and 50 ul <sup>125</sup>I-TSP (10 ng/ml) in a final volume of 200 ul. Tubes containing only tracer and buffer gave the level of non-specific binding (generally 1 - 5%) while the binding of tracer to antibody in the absence of added TSP was also routinely assessed (generally 25 - 60%). After incubation overnight at 20°C, 50 ul Sepharose 4B-coupled donkey anti-rabbit IgG solution was added and the tubes incubated with shaking for 45 min at 20°C. The solid and liquid phases were separated by sedimentation of the Sepharose-bound material through assay buffer containing 10% w/v

sucrose and removal of the supernatant by aspiration (Hunter 1977). Radioactivity was measured in a model NE1600 gamma counter (Nuclear Enterprises Ltd., Edinburgh). The tubes were measured until 50 ul tracer gave 10000 counts. Values of TSP in samples were read from a standard curve which was constructed using doubling dilutions of a human platelet TSP solution covering an approximate range of 1 - 400 ng/ml.

#### 2.4 Radioimmunoassays for vWF and BTG

vWF and BTG were measured by Ms O. Drummond and Ms S. Maguire, Blood Components Assay Group, Edinburgh, by the radioimmunoassays described elsewhere (McArthur et al 1986 and Bolton et al 1976).

#### 2.5 Radioimmunoassay for epidermal growth factor (EGF)

EGF in breast cyst fluid was measured by Dr K. Smith, Division of Oncology, University of Newcastle Upon Tyne, using the radioimmunoassay described below.

Purified recombinant human B-urogastrone (a gift from Dr H. Gregory, ICI Ltd., Macclesfield, Cheshire) was used as standard and iodinated by the Iodogen method for use as tracer. Primary antibody was monoclonal mouse anti-human urogastrone (G.D.Searle, High Wycombe, Bucks) which had been desalted over a Sephadex G-50 Fine column (2.2 x 39 cm) using 0.04 M sodium phosphate, 0.15 M NaCl, 0.01 M EDTA, 0.5% BSA, 0.1% thiomersal, pH 7.2 (buffer A).

Assays were performed in triplicate in Eppendorf tubes in 400 ml buffer A containing 100 ul primary antibody (1:9000 dilution), 100 ul  $^{125}\text{I}$ -urogastrone (80 - 120 uCi/ug; approximately 10000 cpm)

and either unlabelled urogastrone or varying amounts of breast cyst fluid. After incubation for 24 h at 4°C, 100 ul SAC-CEL (donkey anti-mouse) was added, the tubes vortexed, 900 ul distilled water added and left for 45 min at 20°C. The contents were centrifuged at 14000 g for 6 min at 4°C, supernatants discarded and the tips of the Eppendorf tubes cut off and counted in an LKB Wallac 1272-Clinigamma-Spectrometer.

## 2.6 Collection of plasma samples for assays of TSP and BTG

Plasma samples were obtained routinely by collection of venous blood (2.7 ml) into 0.3 ml of ice-cold platelet release inhibitor solution (ETP) containing 78 mM EDTA, 10 mM theophylline and 0.33 ug/ml prostaglandin E<sub>1</sub>, as described by Ludlam and Cash (1976).

## 2.7 Preparation of cytosols from human breast tissue

Buffer (pH 7.6);

20 mM Tris-HCl

0.25 M sucrose

1 mM CaCl<sub>2</sub>

2 mM MgCl<sub>2</sub>

10 mM KCl

Breast tissue from patients attending the Edinburgh Breast Clinic, obtained at the time of mastectomy or biopsy, was dissected free from surrounding fat and connective tissue, and stored at -70°C. The following procedures were carried out on ice or at 4°C.



Cytosols were prepared in the Department of Clinical Surgery, Edinburgh. A piece (100 - 200 mg) of frozen tissue was placed in a centrifuge tube, diced with scissors, and buffer added at a ratio of 1:5 or 1:10 w/v. Tissue was homogenised using a mixer/emulsifier (Silverston Machines Ltd., Chesham, Bucks) at full speed for 20 sec. The homogenate was centrifuged at 105000 g for 1 h at 4°C and the supernatant (cytosol) stored at -20°C.

## 2.8 Anti-TSP enzyme-linked immunosorbant assay (ELISA)

An ELISA was developed to detect murine antibodies against TSP, which was used to screen and monitor the production of monoclonal antibodies by hybridoma cultures.

Wash buffer;

15 mM phosphate buffer, pH 7.4

150 mM NaCl

0.05% Tween 20

Assay buffer;

50 mM phosphate buffer, pH 7.4

150 mM NaCl

0.05% Tween 20

Blocking buffer;

50 mM phosphate buffer, pH 7.4

150 mM NaCl

0.1 % BSA

TMB substrate;

0.01% 3,3',5,5'-tetramethylbenzidine

0.005%  $H_2O_2$

in 100 mM sodium acetate/citrate buffer, pH 6.0

Flat-bottomed 96-well polystyrene microtitre plates (M29AR; Sterilin) were coated overnight at 4°C with 100 ul/well of human platelet TSP (5 - 10 ug/ml in 50 mM phosphate buffer, 0.15 M NaCl, pH 7.4) purified by heparin-Sepharose affinity chromatography. Only the central 60 wells were used to avoid the high non-specific binding which could occur in the edge wells of some plates.

Coated plates were washed 4 x with 250 ul/well wash buffer and incubated with 200 ul/well blocking buffer in a humid chamber for 2 h at 20°C. The plates were washed once with 250 ul/well wash buffer.

Assay buffer (100 ul/well) was added to all wells in the first row (negative control) and duplicate samples (100 ul/well) added to the remaining wells. The plates were incubated in a humid chamber at 20°C overnight.

The plates were washed 4 x as before and incubated with 200 ul/well peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO Ltd., High Wycombe, Bucks) diluted 1/500 in assay buffer containing 0.1% BSA, in a humid chamber for 1 h at 37°C.

The plates were washed 4 x as before and then incubated with 200 ul/well TMB substrate solution for 10 min at 20°C, with shaking. The reaction was stopped by the addition of 50 ul/well 2.5 M  $H_2SO_4$  and shaking continued for a few seconds.

The absorbance of the wells at 450 nm was measured using a

Titertek Multiskan MCC/340 photometer (Flow) which had been blanked on air, and the results expressed as absorbance units after subtraction of the mean negative control value.

## 2.9 Slot-immunobinding assay (SIBA)

Immobilon PVDF transfer membrane (Millipore (UK) Ltd., Harrow, Middlesex) was cut to size (3.8 x 12.4 cm), wet in methanol for 1 - 2 sec and soaked in deionised water. It was placed in a Hybri-Slot manifold (Gibco Ltd., Uxbridge, Middlesex) connected to a vacuum source.

Antigen solutions were applied into the sample wells and filtered under negative pressure. The membrane was removed from the manifold and washed in deionised water before incubation in 20 mM Tris-HCl, 0.9% NaCl, 5% BSA, pH 7.4 (5% BSA-TBS) for 1 h at 37°C to block excess protein-binding sites.

After washing in 0.1% BSA-TBS (3 changes of 5 min each), the membrane was incubated in 1st antibody solution (murine monoclonal anti-TSP diluted in 1% BSA-0.05% Tween 20-TBS) for 2 h at 20°C.

The membrane was washed in 0.1% BSA-TBS (3 x 5 min) and incubated in 2nd antibody solution (peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO) diluted 1/400 in 1% BSA-0.05% Tween 20-TBS) for 2 h at 20°C.

After washing again, bound antibody was visualised by incubating the membrane in 0.02% 3-amino-9-ethylcarbazole, 0.03%  $H_2O_2$  in 0.05M acetate buffer, pH 5.0 (prepared and filtered immediately before use) for 15 min at 20°C. The membrane was rinsed in deionised water, fixed in 3.7% formaldehyde in 0.1 M phosphate buffer, pH 7.5 for 5 min, rinsed again and air dried.

## 2.10 Immunocytochemical techniques

### 2.10.1 Indirect immunoperoxidase (Nakane and Pierce 1966)

Pre-fixed paraffin-embedded sections were de-waxed in 3 changes of CNP 30 and brought to water through graded alcohols (100%, 90%, 70% ethanol). Fresh cryostat sections were air dried or post-fixed in acetone (20 min).

Endogenous peroxidase was blocked by immersing slides in 3%  $\text{H}_2\text{O}_2$  in methanol for 30 min followed by washing in 0.05 M Tris-HCl, 0.85% NaCl, pH 7.6 (TBS) for 5 min.

Possible background reaction was blocked by incubating sections with a layer of normal rabbit serum diluted 1/5 in TBS (NRS-TBS), in a humid chamber for 10 - 15 min at 20°C. The serum was drained off and the slides wiped dry except for areas of section.

Sections were incubated with 1st layer antibody (e.g. murine monoclonal anti-TSP) diluted in NRS-TBS, in a humid chamber for 30 - 60 min at 20°C or overnight at 4°C. Slides were washed in TBS (3 x 5 min) and then dried except for the areas of section.

Sections were incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO) diluted 1/20 in NRS-TBS, in a humid chamber for 30 min at 20°C. Slides were washed in TBS (3 x 5 min) and in 0.05 M Tris-HCl, pH 7.6 (5 min).

Localisation of peroxidase was carried out by incubating slides in 0.05 M Tris-HCl, pH 7.6 containing 0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Aldrich Chemical Co. Ltd., Gillingham, Dorset) and 0.03%  $\text{H}_2\text{O}_2$  for 5 - 20 min (Graham and Karnovsky, 1966). Development of the stain was checked microscopically and optimal staining (dark brown against a light background) was produced after

5 - 20 min.

The reaction was stopped by rinsing slides in deionised water. They were then counterstained lightly with haematoxylin, dehydrated through graded alcohols, cleared in CNP 30 and mounted in DPX.

#### 2.10.2 Indirect peroxidase anti-peroxidase (PAP) (Sternberger 1979)

Sections were processed as for the indirect immunoperoxidase method up to and including blocking of endogenous peroxidase. Slides were then washed in 2% normal swine serum diluted in TBS (3 x 5 min).

Possible background reaction was blocked by incubating sections with a layer of normal swine serum (1/5 in TBS) in a humid chamber for 30 min at 20°C. Slides were drained and wiped dry except for the areas of section.

Sections were incubated with 1st layer antibody (e.g. rabbit anti-TSP) diluted in 2% NSS-TBS, in a humid chamber for 30 min at 20°C or overnight at 4°C. They were washed in 2% NSS-TBS (3 x 5 min) and wiped dry except for the areas of section.

Sections were incubated with 2nd layer antibody (swine anti-rabbit immunoglobulins; DAKO) diluted 1/30 in 2% NSS-TBS, in a humid chamber for 30 min at 20°C. They were washed in 2% NSS-TBS (3 x 5 min) and wiped dry except for the areas of section.

Sections were incubated with peroxidase-rabbit anti-peroxidase (DAKO) diluted 1/100 in 2% NSS-TBS, in a humid chamber for 30 min at 20°C. They were washed in 2% NSS-TBS (3 x 5 min) and in 0.05 M Tris-HCl, pH 7.6 (5 min).

PAP was localised by incubating the slides in DAB solution as

for the indirect immunoperoxidase method. Optimal staining was obtained within 1 - 5 min, after which the slides were rinsed, counterstained, dehydrated, cleared and mounted as before.

## 2.11 Cell culture

### 2.11.1 Human endothelial cells

#### 2.11.1.1 Isolation

Growth medium (complete);

Medium 199 (modified)

with Earle's salts

20 mM HEPES

20% v/v human serum

2 mM L-glutamine

0.4 g/l  $\text{NaHCO}_3$

0.15 g/l endothelial cell growth supplement

(ECGS; Maciag et al 1979)

100 U/ml penicillin

100 mg/ml streptomycin

2.5 ug/ml Fungizone<sup>R</sup> (Flow)

Growth medium (serum-free; deGroot et al 1983);

Medium 199 (modified)

with Earle's salts

20 mM HEPES

non-essential amino acids (NEAA)

2 mM L-glutamine

0.4 g/l  $\text{NaHCO}_3$

0.15 g/l ECGS

10 ng/ml epidermal cell growth factor (EGF)

1% w/v BSA

10 ug/ml insulin

20 ug/ml transferrin

100 U/ml penicillin

100 mg/ml streptomycin

2.5 ug/ml Fungizone<sup>R</sup>

Endothelial cells were isolated from human umbilical cords obtained from the Maternity Wards of the Western General Hospital, Edinburgh or the Simpson Maternity Pavilion, Edinburgh. The cords were placed in glass bottles containing 50 ml Medium 199 (Flow), 200 U/ml penicillin-G potassium salt, 200 ug/ml streptomycin sulphate and stored at 20°C for up to 12 h after delivery.

Damaged or overdistended portions of the cord were excised and the required vessel was cannulated. For human umbilical vein endothelial cells (HUVEC) the umbilical vein was cannulated using a 16 gauge catheter, while for human umbilical artery endothelial cells (HUAEC) one umbilical artery was cannulated using a 1 mm rounded-end capillary tube. In either case the cannula was secured

with silk thread.

The vessel was perfused with 20 - 50 ml Earle's buffered salt solution (Flow) to remove blood and clots, then with a 1 mg/ml solution of collagenase, type IA (Sigma) in Medium 199 (for the artery, 10 ug/ml Trapidil<sup>R</sup>, a potent vasodilator, was added to both solutions). The effluent end was tied off, more enzyme solution infused to fill the vessel, the affluent end tied off and the cord wrapped in aluminium foil. After incubation at 37°C for up to 15 min, the cord was massaged gently to aid dislodgement of cells from the vessel lumen. The vessel was perfused with 20 ml Medium 199, the effluent collected and centrifuged at 200 g for 10 min at 15°C. The pellet was resuspended in 5 ml complete medium (containing 30% v/v human serum) and transferred into a 25 cm<sup>2</sup> tissue culture flask which had been pre-coated with 10 ug/cm<sup>2</sup> human fibronectin (Scottish National Blood Transfusion Service Protein Fractionation Centre).

After 4 h the medium was replaced with complete medium (30% v/v human serum) and replaced every 48 h thereafter with complete medium (20% v/v human serum) until the cells reached confluence.

#### 2.11.1.2 Subculturing

At confluence, the cells were subcultured with a 1:4 split ratio as follows. The monolayer was washed with buffered salt solution to remove serum components and incubated with 2 ml/25 cm<sup>2</sup> of a solution containing 0.025% trypsin (Flow) and 0.020% EDTA in phosphate buffered salts, Dulbecco's formula (modified) without Ca and Mg, using agitation to release the cells (usually complete within 5 min). An equal volume of complete medium was added to



inhibit trypsinisation and the cell suspension transferred into sterile universal containers for centrifugation at 200 g for 5 min. The supernatant was decanted, the container flicked vigorously to disperse the pellet and the cells resuspended in a suitable volume of fresh complete medium for transfer into a pre-coated flask, or in freezing medium for preservation.

#### 2.11.1.3 Preservation

To the dispersed cell pellet from a trypsinised culture, 1 ml cold freezing medium (90% v/v complete medium / 10% v/v dimethyl sulphoxide, DMSO) was added dropwise with gentle shaking. The resulting cell suspension was transferred aseptically into a sterile screw-top plastic vial and cooled at 1°C/min in the vapour phase of liquid nitrogen for at least 1 h. Frozen cells were stored in the vapour phase of a liquid nitrogen container.

For recovery of frozen cells, a vial was thawed in a water bath at 37°C. The outside of the vial was sterilised with alcohol and the contents transferred aseptically to a pre-coated flask containing fresh complete medium (the DMSO should be diluted at least 10-fold). Medium was replaced after 24 h, the flask reincubated, and medium replaced every 48 h thereafter.

### 2.11.2 Human fibroblasts

Growth medium (complete);

RPMI 1640

20 mM HEPES

10% v/v human serum

NEAA

4.0 mM L-glutamine

0.4 g/l  $\text{NaHCO}_3$

100 U/ml penicillin

100 mg/ml streptomycin

2.5 ug/ml Fungizone<sup>R</sup>

Human oral fibroblasts (HOF) or human skin fibroblasts (HSF) (generous gifts from Dr Y. Barlow and Dr G.C. Priestley, Department of Dermatology, Royal Infirmary, Edinburgh) were cultured essentially as described for human endothelial cells.

### 2.11.3 MCF-7 Human breast cancer cells

Growth medium (complete);

Eagle's Minimal Essential Medium

with Hank's salts (Flow)

20 mM HEPES

10% v/v foetal calf serum (Gibco)

NEAA

2.0 mM L-glutamine

1.0 mM sodium pyruvate

0.35 g/l  $\text{NaHCO}_3$

10 ug/ml insulin

Growth medium (serum-free);

Ham's F12/DMEM 1:1 v/v (Flow)

15 mM HEPES

0.386 g/l L-glutamine

1.2 g/l  $\text{NaHCO}_3$

10 nM  $\text{Na}_2\text{SeO}_4$

250 ng/ml insulin

25 ug/ml transferrin

10 ng/ml EGF

100 ng/ml prostaglandin  $\text{F}_{2\alpha}$

7.5 ug/ml fibronectin

Freezing medium;

95% v/v complete medium / 5% v/v DMSO

MCF-7 Human breast cancer cells were obtained from Professor S.C. Brooks, Michigan Cancer Foundation, Detroit, USA. This cell line was derived from a pleural effusion from a patient with breast adenocarcinoma, and retains several characteristics of differentiated mammary epithelium (Soule et al 1973). Cells were maintained in routine culture as recommended, and for some experiments they were transferred into serum-free culture (Barnes and Sato 1979).

#### 2.11.4 Detection of mycoplasma (Chen et al 1977)

McIlvaine's buffer (pH 5.5);

11.37 ml 0.2 M  $\text{Na}_2\text{HPO}_4$

8.62 ml 0.1 M citric acid

Glass coverslips were seeded with cells which had been grown in the absence of antibiotics. The cells were fed 24 h prior to fixation in 3 ml methanol:acetic acid (3:1 v/v) added dropwise to the complete medium, and left for at least 1 min. This was aspirated and replaced with 5 ml fresh fixative solution and incubated a further 5 min. After washing with fixative and drying in air, the cells were incubated with 5 ml 0.05 ug/ml bisbenzimidide (Hoechst 33258) in phosphate buffered salt solution (containing Ca and Mg) for 10 min. The cells were washed twice with distilled water and the coverslips mounted in McIlvaine's buffer and sealed. The stain was visualised under a fluorescent microscope.

## 2.12 Production of monoclonal antibodies against TSP

### 2.12.1 Immunisation

Mice (female Balb/c, 12 - 15 weeks old; bred in the Medical Faculty Animal Facility, Edinburgh from stock originally obtained from the Laboratory Animal Centre, Carshalton) were primed by intraperitoneal injection of a 1:1 v/v mixture of human platelet TSP (50 ug/mouse) and Freund's complete adjuvant, then boosted after 6 - 9 weeks with 50 ug TSP in aluminium hydroxide gel (Superfos Speciality Chemicals a/s, Vedbaek, Denmark) and after 19 - 30 weeks with 50 - 100 ug TSP in Dulbecco 'A' saline.

Mice were bled from the tail 10 - 14 days post-boosting and the sera tested for antibodies binding  $^{125}\text{I}$ -TSP in a modified radioimmunoassay. Briefly, tubes containing 50 ul each of serum (doubling dilutions from 1/100 - 1/3200), assay buffer and tracer were incubated overnight, and then with 50 ul Sepharose 4B-sheep anti-mouse immunoglobulins (1:1 v/v;; prepared according to section 2.3.3 using antiserum from the Scottish Antibody Production Unit) for 45 min with shaking. The bound and free tracer were separated by sedimentation through 10% sucrose. Mice which showed a positive antigen-binding curve were sacrificed and their spleens removed.

### 2.12.2 Hybridisation (Micklem et al 1987)

Basic medium (RPMI-15FCS);

RPMI 1640 (Gibco)

20 mM HEPES

15% foetal calf serum

2.0 mM L-glutamine

1.0 mM sodium pyruvate

50 U/ml penicillin

50 U/ml streptomycin

HT medium;

RPMI-15FCS plus

0.1 mM hypoxanthine

16 uM thymidine

HAT medium;

HT medium plus

0.4 uM aminopterin

Spleen cells were fused with NS-0 mouse plasmacytoma cells (Galfre and Milstein 1981) in a 1:1 cell ratio using 50% PEG 4000 at pH 8.5 - 9.0 (Kennett et al 1978). Fused cells were seeded in Costar 96-well tissue culture clusters (Northumbria Biologicals, Cramlington, Northumberland) at  $2 \times 10^5$  cells/well in 0.1 ml HAT medium/well and incubated at 37°C in 5% CO<sub>2</sub>/air. The wells had previously been charged with 0.1 ml mixed-thymocyte conditioned medium (MTM) diluted 1:2 v/v with HAT medium. When sufficiently

well grown, hybrids were tested for antibody secretion by ELISA (section 2.8), and those which were positive were then transferred to 24-well plates.

#### 2.12.3 Cloning by limiting dilution (Micklem et al 1987)

Cells were diluted in 10% MTM in RPMI-15FCS and plated at 0.1 ml/well in 96-well plates to give (theoretically) 2 rows at 5 cells/well, 2 rows at 1 cell/well and 2 rows at 0.5 cells/well. After incubation for 7 days, the number of clones per well was recorded and 3 drops of RPMI-15FCS were added to each well. Clones were tested for antibody secretion by ELISA when sufficiently well grown. Those which were positive were re-cloned as necessary until a stable cell line (i.e. one which gave 100% positive clones) was achieved in each case.

#### 2.12.4 Bulk production

Monoclonal antibodies were produced in bulk by culture of cloned cell lines in vitro and by growth as ascitic fluid in vivo.

Cells were generally grown up in 150 cm<sup>2</sup> tissue culture flasks (Corning, New York, USA) in RPMI 1640 supplemented with 5% foetal calf serum and the antibody-rich medium harvested at cell death. One cell line (producing antibody ESTs8) was grown up in a CelliGen cell culture system (New Brunswick Scientific (UK) Ltd., Watford, Herts) in RPMI 1640 with 10% foetal calf serum, pH 7.2, dissolved oxygen 30%, stirring rate 30 rpm, at 37°C.

Ascitic fluid was produced as follows. Mice (male Balb/c) were primed with two injections of 0.5 ml Pristane (2,6,10,14-tetramethyl-pentadecane) at an interval of 7 days, up to 14 weeks before



inoculation of cells. The mice were inoculated intraperitoneally with  $3 - 5 \times 10^5$  cells in Dulbecco 'A' saline. Ascitic fluid was tapped at intervals up to 4 weeks after inoculation.

#### **2.12.5 Determination of antibody type**

Monoclonal antibodies were typed by radial immunodiffusion (RID) employing specific anti-mouse Ig subclass antibodies (Monoclonal Antibody Typing Kit RK008; The Binding Site Ltd., Birmingham). Samples of bulk culture supernatants and specific antisera were applied to the wells of the RID plates as directed. After incubation for 24 - 48 h, the plates were observed for precipitation lines which indicated the subclass of the antibodies.

#### **2.12.6 Purification of monoclonal IgG antibodies by affinity chromatography on protein A**

##### **2.12.6.1 Preparation of a protein A-Sepharose 4B column**

Coupling buffer (pH 8.3);

0.1 M  $\text{NaHCO}_3$

0.5 M NaCl

A suspension of 7 g CNBR-activated Sepharose 4B (Pharmacia) in 1 mM HCl was washed with 1.4 l 1 mM HCl in a 100 ml glass conical flask using a filter tube with sintered glass end (Pyrex) connected to a vacuum line. A solution of 250 mg protein A (Fermentech, Edinburgh) in 35 ml coupling buffer was added to the washed gel, the flask stoppered and mixed by inversion overnight at 4°C.



The coupled gel was washed in the flask as before with 400 ml coupling buffer and suspended in 30 ml 1 M ethanolamine, pH 9. After rotation for 2 h at 20°C, the product was washed with 3 cycles of alternating pH. Each cycle consisted of 300 ml 0.1 M acetate buffer, 0.5 M NaCl, pH 4, followed by 300 ml 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.

The product was adjusted to an approximate 75% v/v gel solution in pH 8 buffer and poured into a K26/40 column fitted with two A26 flow adapters (Pharmacia).

#### 2.12.6.2 Elution of monoclonal IgG on protein A-Sepharose 4B

High pH buffer (pH 8.5);

0.1 M  $\text{Na}_2\text{HPO}_4$

0.1 M trisodium citrate

2.0 M NaCl

Low pH buffer (pH 2.0);

0.1 M citric acid

Dialysis buffer (pH 6.0);

50 mM  $\text{NaH}_2\text{PO}_4$

50 mM trisodium citrate

Bulk culture supernatant was concentrated 20 x by ultrafiltration (Amicon), passed through a pre-filter (type AP20; Millipore), NaCl added to a final concentration of 2.0 M and the pH adjusted to 8.5 with 1 M NaOH. Ascites fluid was centrifuged at

15000 g to remove particulate material, NaCl added and the pH adjusted as above.

The column was equilibrated in high pH buffer and the sample loaded at 0.8 - 0.9 ml/min using a peristaltic pump P-1 (Pharmacia), followed by washing with high pH buffer and monitoring the absorbance of the eluate at 280 nm. When the baseline had returned to zero, the column was eluted with a linear gradient formed from 120 ml high pH buffer and 120 ml low pH buffer using a gradient mixer GM-1 (Pharmacia) and collecting 80 x 3 ml fractions in a fraction collector FRAC-100 (Pharmacia).

Each fraction was assayed for protein ( $OD_{280}$ ), pH and mouse IgG (by radial immunodiffusion, section 2.12.5); fractions which were positive by  $OD_{280}$  and RID were pooled, neutralised and dialysed overnight. The sample was filtered (0.2  $\mu$ m) and concentrated, if necessary, until the  $OD_{280}$  reached 1.4 - 1.5. Maltose was added (10% w/v) and the sample stored in aliquots at -20°C or lyophilised.

#### 2.12.7 Detection of monoclonal IgG by radial immunodiffusion

Aronson and Grunwall buffer, pH 8.9;

100 mM Tris

4 mM EDTA, free acid

15 mM boric acid

Radial immunodiffusion plates were prepared as follows. Agarose (1 g; Miles Laboratories Ltd., Slough, Berks) was boiled in 100 ml Aronson and Gronwall buffer until clear, and cooled to 56°C. Sheep

anti-mouse IgG (1 ml; Scottish Antibody Production Unit) was mixed with 2<sup>ml</sup> agarose solution, poured into a 10 cm<sup>2</sup> plastic petri dish (Sterilin) on a level table and allowed to set. Wells were made with a 2 mm well cutter (10 mm apart, 81 wells/plate), samples applied (10 ul/well) and the plate incubated overnight at 20°C. Observation of precipitate rings indicated the presence of mouse IgG antibody in the sample.

#### **2.12.8 Purification of monoclonal IgM antibodies by gel filtration on Sephacryl S-300**

Bulk culture supernatant (1000 ml) was concentrated 100 x by ultrafiltration (Amicon) and glucose added (1% w/v) to increase the sample density. The sample was applied under the eluent onto a 2.5 x 78 cm Sephacryl S-300 gel filtration column (Pharmacia) which was equilibrated and eluted with 0.4% w/v trisodium citrate, 0.15 M NaCl, pH 7 buffer. The flow rate was 0.8 - 0.9 ml/min using a P-1 pump (Pharmacia) and 90 x 8 ml fractions were collected in a FRAC-100 fraction collector (Pharmacia).

Each fraction was assayed for protein (by absorbance at 280 nm) and the presence of anti-TSP antibody (by ELISA). Those fractions which were positive by ELISA were pooled and concentrated to 50 ml as before. Maltose (10% w/v) was added, and the sample filtered through a AP20 pre-filter (Millipore) and through a Sterivex-GS 0.22 um filter unit (Millipore) prior to storage in aliquots at -20°C or lyophilisation.

## CHAPTER THREE

### THROMBOSPONDIN IN PLASMA AND SERUM

Studies of TSP in plasma and serum were done on three groups of patients (general surgery, Christmas disease and thrombocytopenic) and in an animal model (dogs receiving infusions of blood products). The aim of this work was to investigate the factors which influence circulating TSP levels, as far as was possible within the confines of these larger clinically-orientated studies. Measurements of BTG and vWF were sometimes made alongside those of TSP, as indicators of specific  $\alpha$ -granule release and of possible endothelial cell activation respectively.

#### 3.1 Patients undergoing general surgery

Plasma samples were taken from 20 patients; age (mean  $\pm$  SD) = 39  $\pm$  7.4 y, range = 24 - 51 y; having general surgical procedures, before anaesthesia and at 30 min, 60 min, 24 h and 96 h intervals post-operatively.

##### 3.1.1 Comparison of pre-operative levels of TSP, BTG and vWF with those in normal plasma

Table 3-1 shows the plasma levels of TSP, BTG and vWF measured before anaesthesia, compared with those in normal individuals previously measured in this laboratory (Dawes et al 1983). No statistically discernible differences were observed in the plasma

Table 3-1. Comparison of pre-operative plasma levels of TSP, BTG and vWF with those in normal plasma

		Pre-op	Normal
TSP	mean $\pm$ SD	111.3 $\pm$ 123.9	105.0 $\pm$ 31.0
	range	28 - 600	57.5 - 215.6
	N	20	27
BTG	mean $\pm$ SD	59.6 $\pm$ 58.6	37.2 $\pm$ 10.9
	range	12.8 - 237.5	20.1 - 68.5
	N	20	27
vWF	mean $\pm$ SD	1.37 $\pm$ 0.48	no data
	range	0.53 - 2.40	
	N	20	

Results expressed in ng/ml (TSP and BTG) and iu/ml (vWF).

concentrations of TSP or BTG in the two groups (Student's t test). No data was available for vWF in normal individuals.

### **3.1.2 Comparison of pre and post-operative plasma levels of TSP, BTG and vWF**

Plasma levels of TSP, BTG and vWF measured before anaesthesia and at intervals post-operatively are shown in fig. 3-1. No statistically discernible differences (Student's t test) were observed between the pre and post-operative concentrations of TSP nor BTG, which were both relatively constant.

However, for vWF the plasma content was observed to increase after surgery in all the patients studied. The highest levels occurred at either 24 h or 96 h post-operatively, when they became statistically discernible from the level before anaesthesia ( $P < 0.001$ , Student's t test).

### **3.1.3 Relationships between plasma TSP, BTG and vWF levels**

The relationship between TSP and BTG for all plasma samples taken before and after surgery is shown in fig.3-2. A positive correlation ( $r = 0.81$ ) which was statistically discernible ( $P < 0.005$ ) was observed between the two.

The relationship between TSP and vWF for the same samples is shown in fig.3-3. A lack of correlation between the two was apparent ( $r = 0.16$ ) and this was confirmed by statistical testing ( $P > 0.05$ ).

**Fig. 3-1. Plasma TSP, BTG and vWF levels after general surgery**

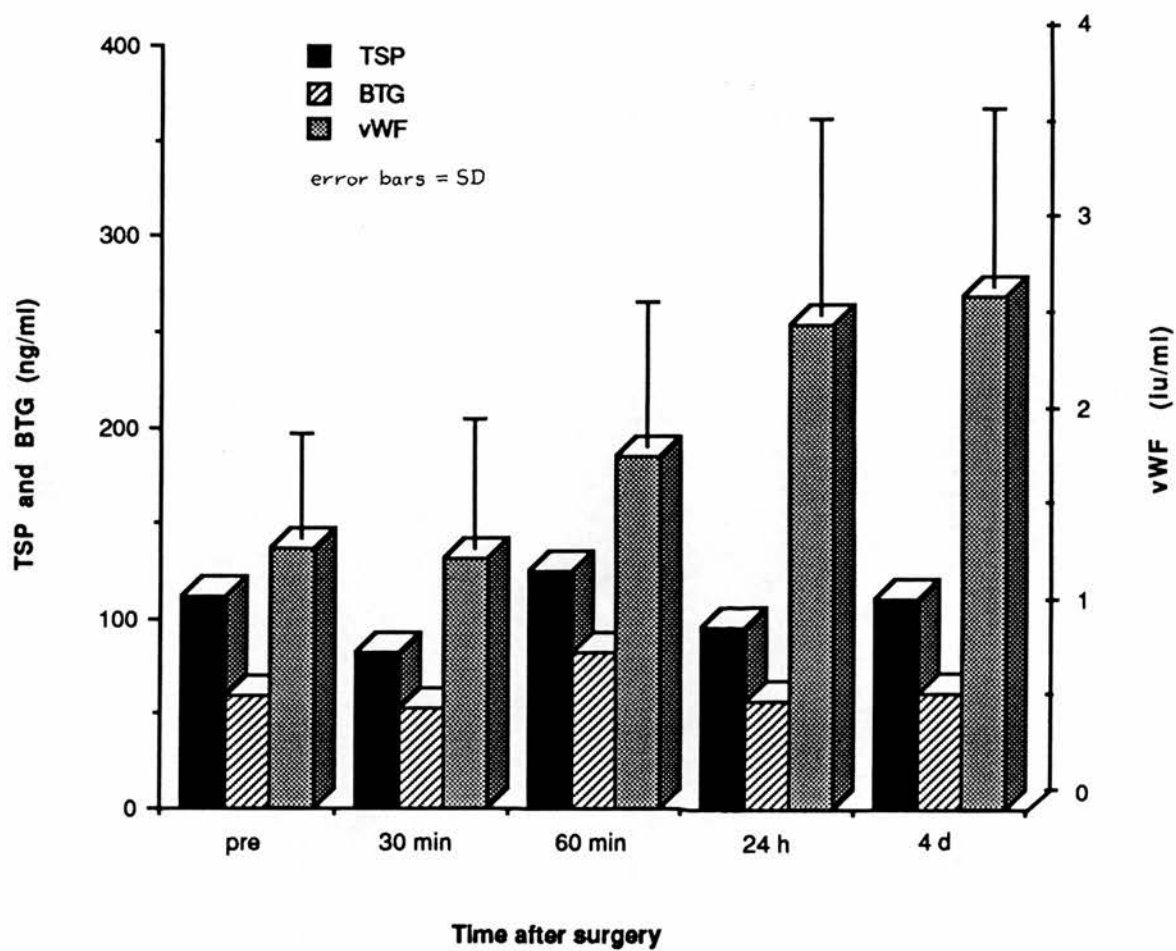


Fig. 3-2. Relationship between plasma TSP and BTG in general surgery patients

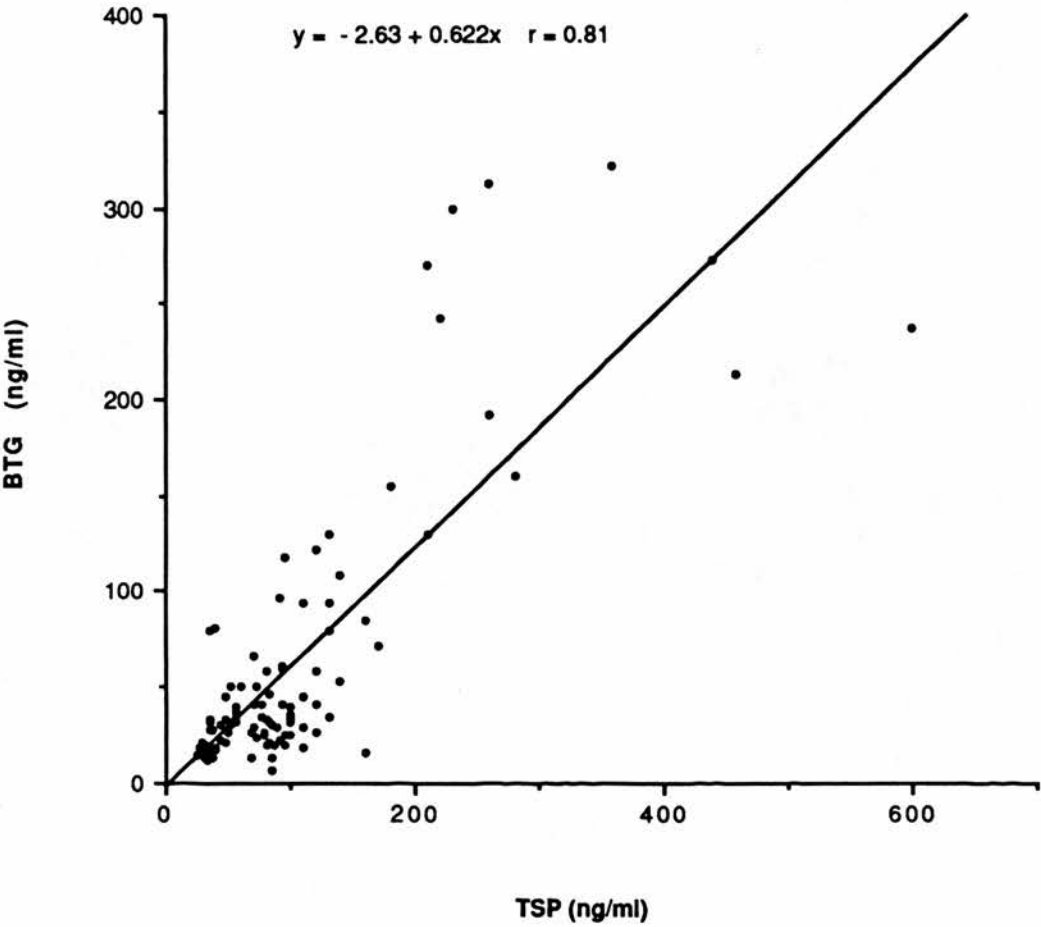
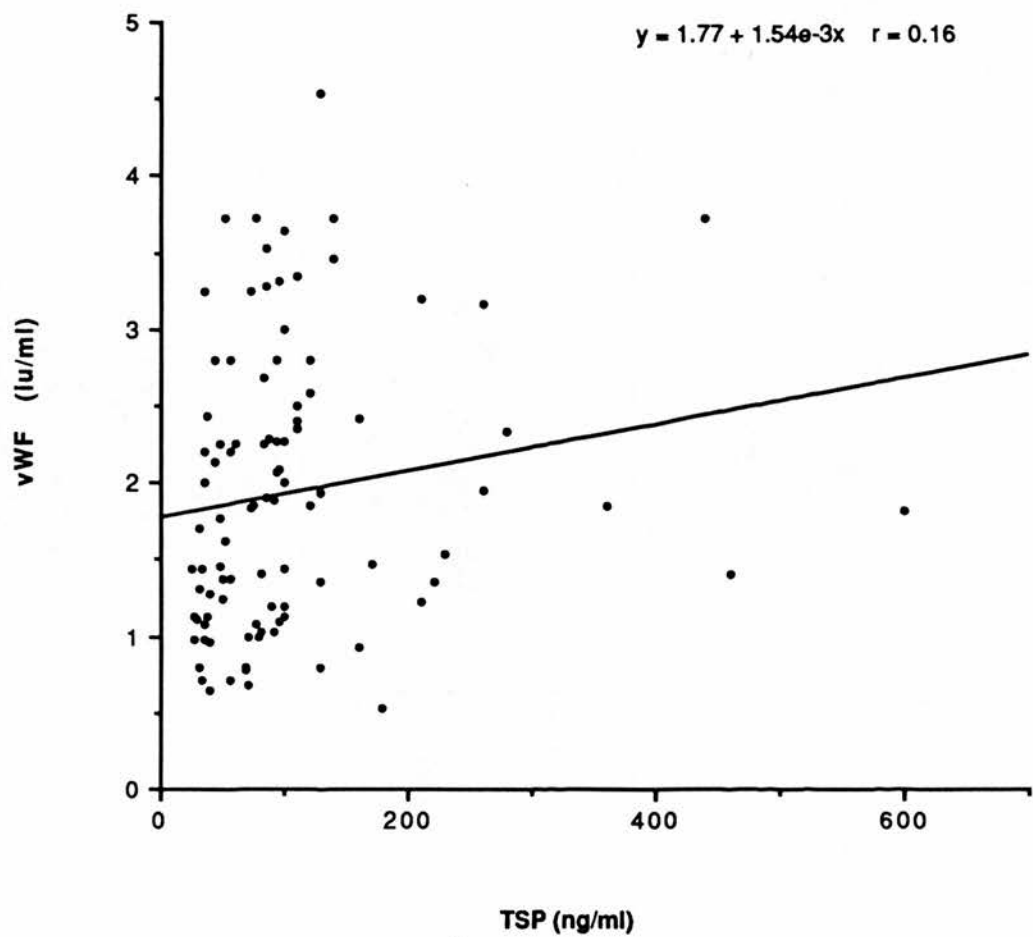




Fig. 3-3. Relationship between plasma TSP and vWF in general surgery patients



#### 3.1.4 Comparison of plasma TSP, BTG and vWF levels after general or epidural anaesthesia

Plasma TSP, BTG and vWF were measured as before in two groups of patients undergoing general surgical procedures. One group ( $n = 5$ , age =  $38 \pm 5.6$  y, range = 33 - 47 y) had a general anaesthetic, while the other ( $n = 8$ , age =  $38 \pm 8.8$  y, range = 22 - 51 y) had an epidural anaesthetic. The results are summarised in table 3-2. Statistical comparisons (Student's t test) between the two groups of patients did not reveal any discernible differences in the plasma levels of TSP, BTG or vWF in samples taken at corresponding times. In both groups, vWF increased similarly with time after surgery, and the differences compared with the corresponding levels before anaesthesia became statistically discernible at 24 h and 96 h.

#### 3.2 Christmas disease patients and TSP in human factor IX concentrate

Plasma levels of TSP were studied in three patients with Christmas disease, an hereditary deficiency of clotting factor IX, during treatment by intravenous infusion of factor IX concentrate. Samples were taken antecubitally by separate venepuncture before infusion was commenced and at intervals of 15 min, 2 h, 5 h, 7 h and 24 h after its completion. Details of the patients and infusions are given in table 3-3, and their plasma TSP levels and factor IX activities are shown in fig.3-4.

In all three patients, the initial plasma TSP concentrations were normal and increased to high levels (5 - 9 times normal) immediately post-infusion. The plasma TSP levels fell rapidly

Table 3-2. Plasma TSP, BTG and vWF levels after general or epidural anaesthesia

		TSP	BTG	vWF
GENERAL (N = 5)				
Pre		85.5 ± 39.9	38.0 ± 16.4	1.50 ± 0.47
Post	30 min	40.4 ± 9.6	28.9 ± 6.7	1.52 ± 0.50
	60 min	61.8 ± 27.4	39.7 ± 12.7	2.27 ± 0.89
	24 h	80.8 ± 47.1	45.8 ± 26.8	2.87 ± 0.85 <sup>a</sup>
	96 h	77.4 ± 41.4	26.5 ± 6.8	3.04 ± 1.23 <sup>b</sup>
EPIDURAL (N = 8)				
Pre		64.4 ± 21.0	53.6 ± 55.7	1.28 ± 0.40
Post	30 min	46.1 ± 19.4	38.0 ± 26.0	1.10 ± 0.19
	60 min	90.7 ± 44.7	36.4 ± 23.0	1.96 ± 0.79
	24 h	104.1 ± 16.5	50.2 ± 38.7	2.54 ± 0.79 <sup>c</sup>
	96 h	81.0 ± 17.5	32.5 ± 8.0	2.61 ± 0.53 <sup>d</sup>

Results expressed in ng/ml (TSP and BTG) and iu/ml (vWF).

<sup>a</sup> P < 0.02 (statistically discernible from pre-anaesthesia level).

<sup>b</sup> P < 0.05 (ditto).

<sup>c</sup> P < 0.005 (ditto).

<sup>d</sup> P < 0.001 (ditto).

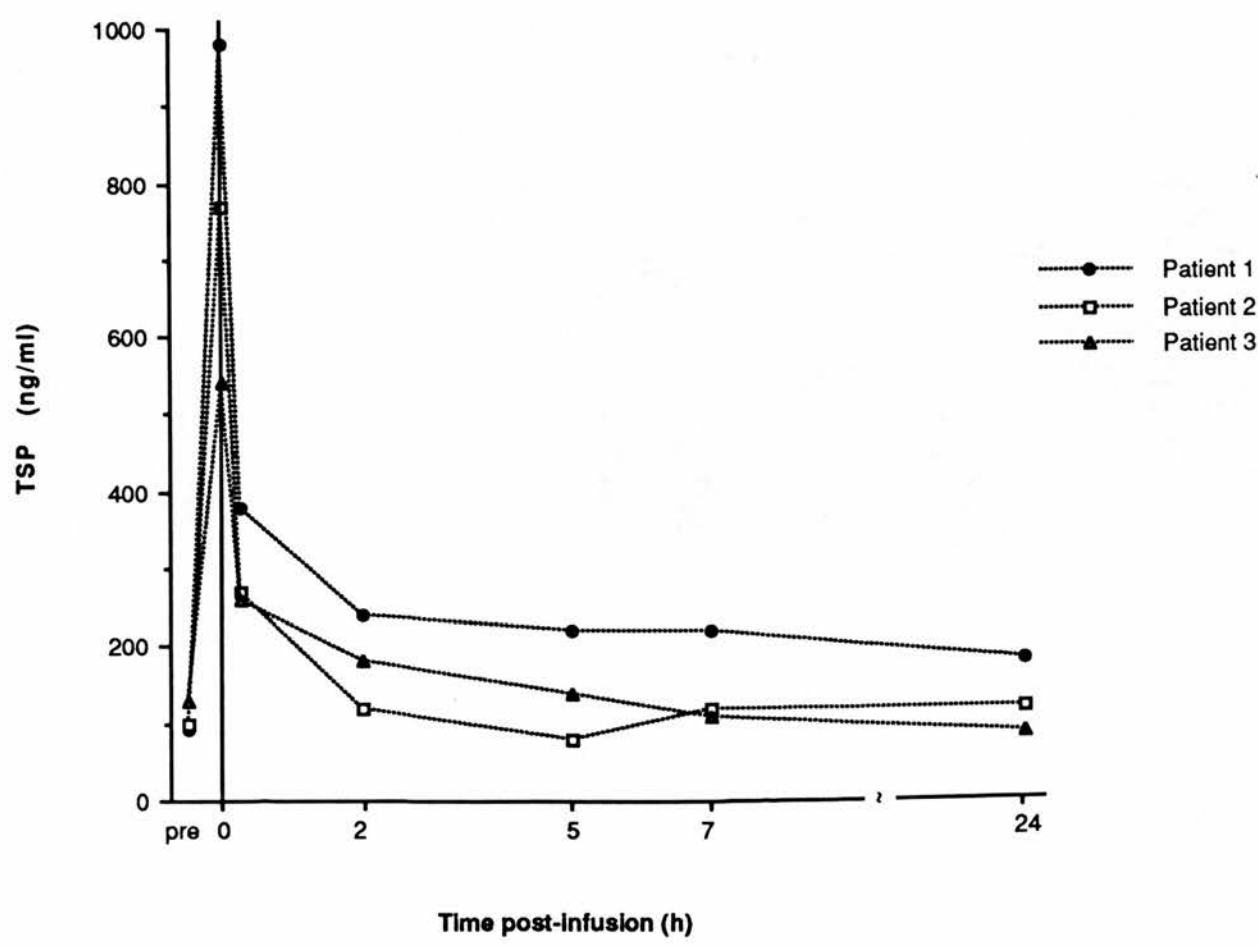
Table 3-3. Christmas disease patients receiving factor IX infusions

Patient	Body weight (kg)	Plasma volume (ml)	Infusion volume (ml)	Plasma TSP increment (ng/ml)	Recovery <sup>*</sup> of TSP (%)
A	54	2310	81	886	23.0
B	90	3780	79	670	29.1
C	79	3530	100	410	13.1

<sup>\*</sup> Recovery of TSP =  $\frac{\text{plasma vol} \times \text{increment}}{\text{infusion vol} \times 110,000} \times 100$

where 110,000 ng/ml = TSP concentration of factor IX infusion

Fig. 3-4. Plasma TSP In Christmas disease patients receiving factor IX Infusions



during the first 15 min, then in an approximately exponential manner up to 5 h post-infusion, when, in two of the patients (B and C) normal levels had been reached, and in the other patient (A) the level was still elevated but falling gradually. The half-life of TSP in plasma during the exponential phase of clearance (15 min - 5 h) was  $148 \pm 55$  min (mean  $\pm$  SD).

In contrast, the plasma factor IX activities in these patients (data provided by Dr C. Prowse, Scottish National Blood Transfusion Service, Edinburgh) reached their highest levels at 15 min post-infusion and then decreased in an approximately linear manner to 24 h, when they were still markedly above those measured prior to infusion of factor IX concentrates (fig.3-5).

The TSP content of lyophilised human factor IX concentrate (Scottish National Blood Transfusion Service Protein Fractionation Centre) was analysed by affinity chromatography using a 2 x 24 cm heparin-Sepharose CL-6B column (Pharmacia). When reconstituted with 20 ml distilled water, the factor IX concentrate contained 8.6 mg/ml protein and 42 ug/ml TSP (0.5% total protein).

Fig.3-6 shows the heparin affinity elution profile obtained from 16 ml factor IX concentrate loaded onto the column (equilibrated in 15 mM Tris-HCl, 0.35 M NaCl, 0.02%  $\text{NaN}_3$ , pH 7.4) and eluted using a stepwise gradient of 0.35 M, 0.55 M and 2.0 M NaCl. Measurement of TSP concentrations in the fractions showed that most of the antigen was contained in the peak eluted with 0.55 M NaCl (fractions 57-60, mean TSP = 38 ug/ml), but some TSP was also detected in the other peaks (e.g. fraction 8, 0.4 ug TSP/ml; fraction 12, 7.4 ug TSP/ml, fractions 70-72, 2.6 ug TSP/ml).

Fig. 3-5. Plasma factor IX In Christmas disease patients receiving factor IX Infusions

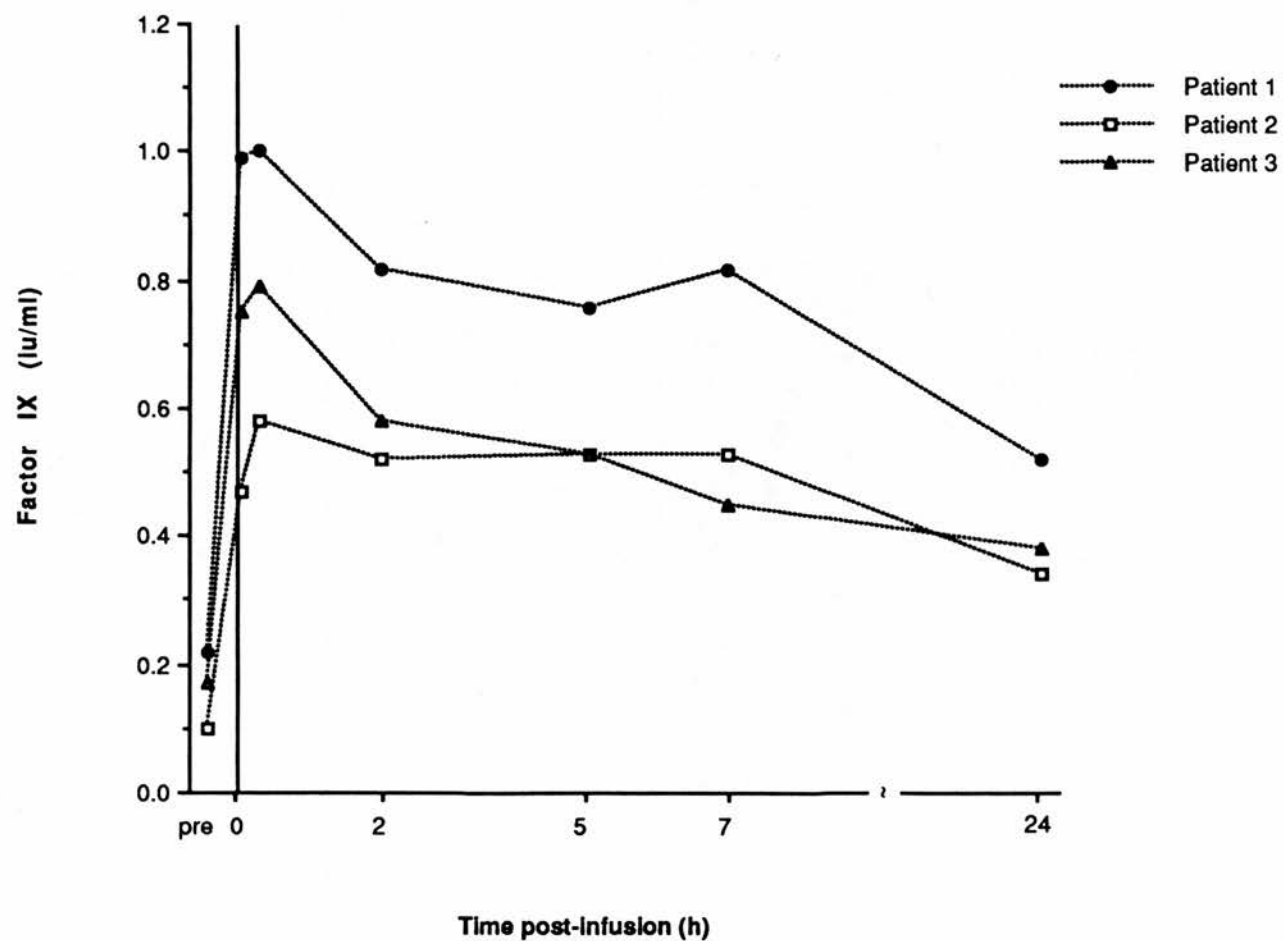
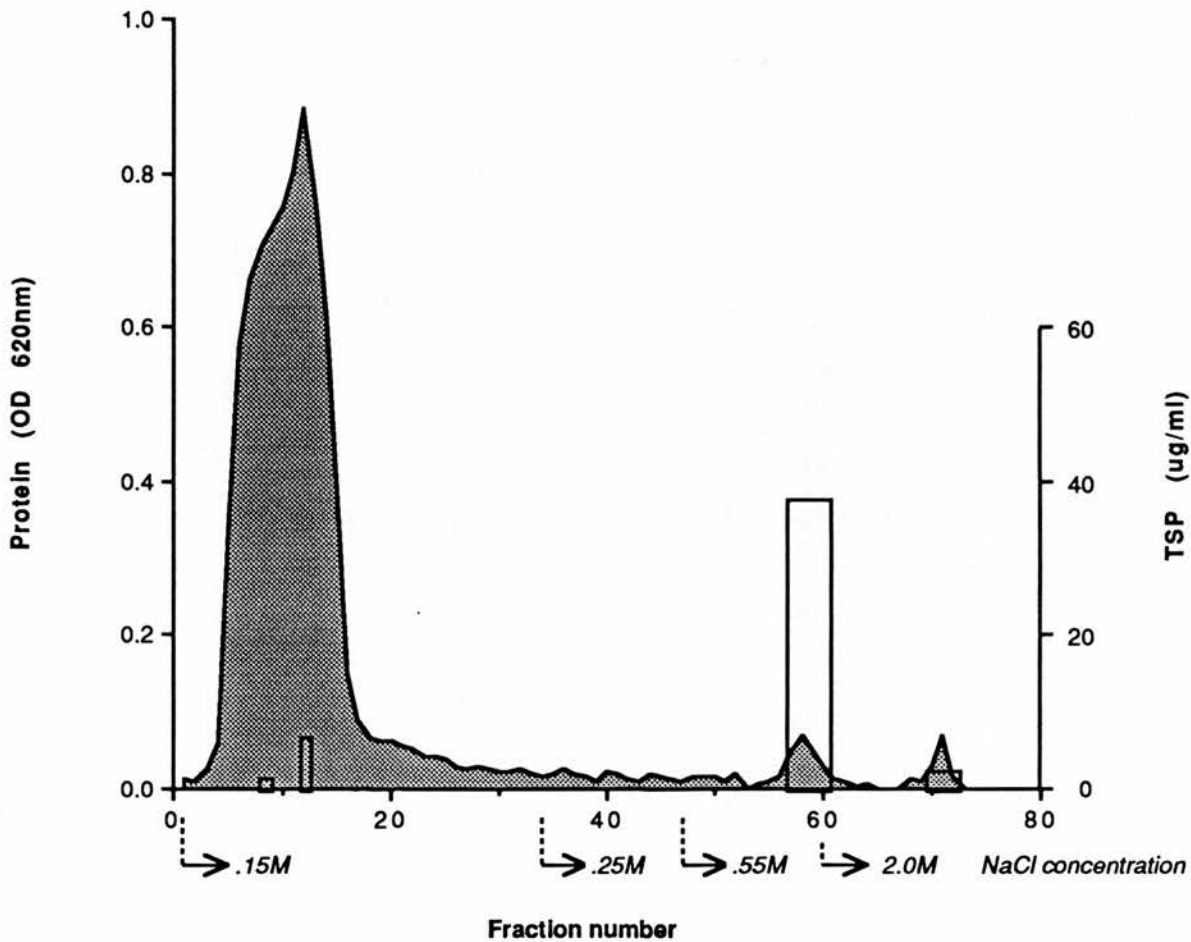


Fig. 3-6. Heparin-Sepharose affinity chromatogram of human factor IX concentrate





### 3.3 Thrombocytopenic patients

TSP and BTG were measured in plasma and serum samples from 20 patients with profound marrow hypoplasia, usually following chemotherapy (table 3-4). In all cases, chemotherapy caused severe peripheral pancytopenia attributable to the marrow hypoplasia. Individual samples were taken prior to any platelet transfusion. In addition, serial samples were taken from three of these patients during recovery from severe bone marrow depression.

#### 3.3.1 Plasma and serum levels of TSP and BTG in severe peripheral pancytopenia

Platelet count, plasma and serum levels of TSP and BTG in 19 patients with severe peripheral pancytopenia are shown in fig.3-7. In every case the platelet count and serum levels of TSP and BTG were less than 10% of normal.

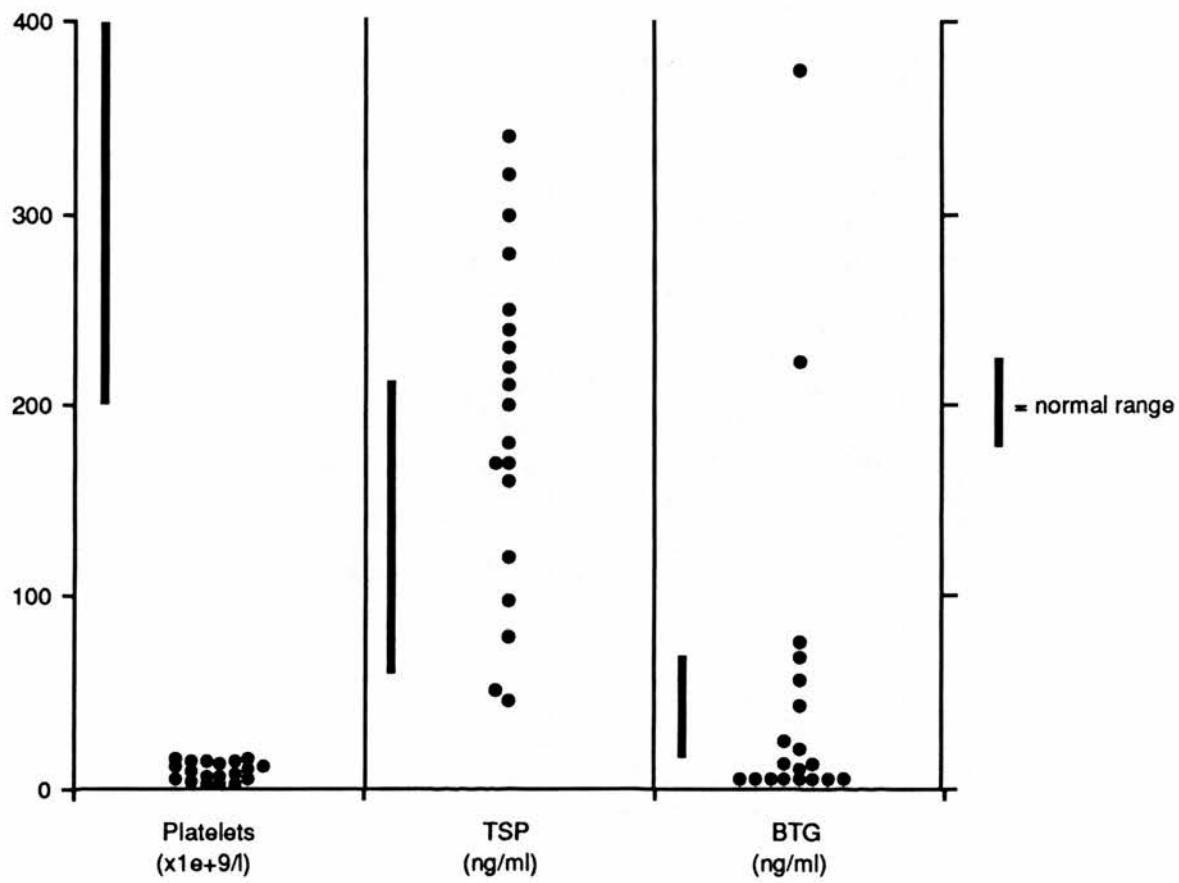
The plasma TSP level was normal or elevated in 17 of the 19 patients (89%), whereas the plasma BTG concentration was below normal in 11/19 (58%) of cases (in 7 of these it was undetectable by the radioimmunoassay).

No statistically discernible relationship was observed between plasma levels of TSP and BTG in patients with severe peripheral pancytopenia ( $r = 0.25$ ;  $P > 0.10$ ), but there was a positive correlation between their serum TSP and BTG concentrations which was statistically discernible ( $r = 0.75$ ;  $P < 0.0005$ ) (fig. 3-8).

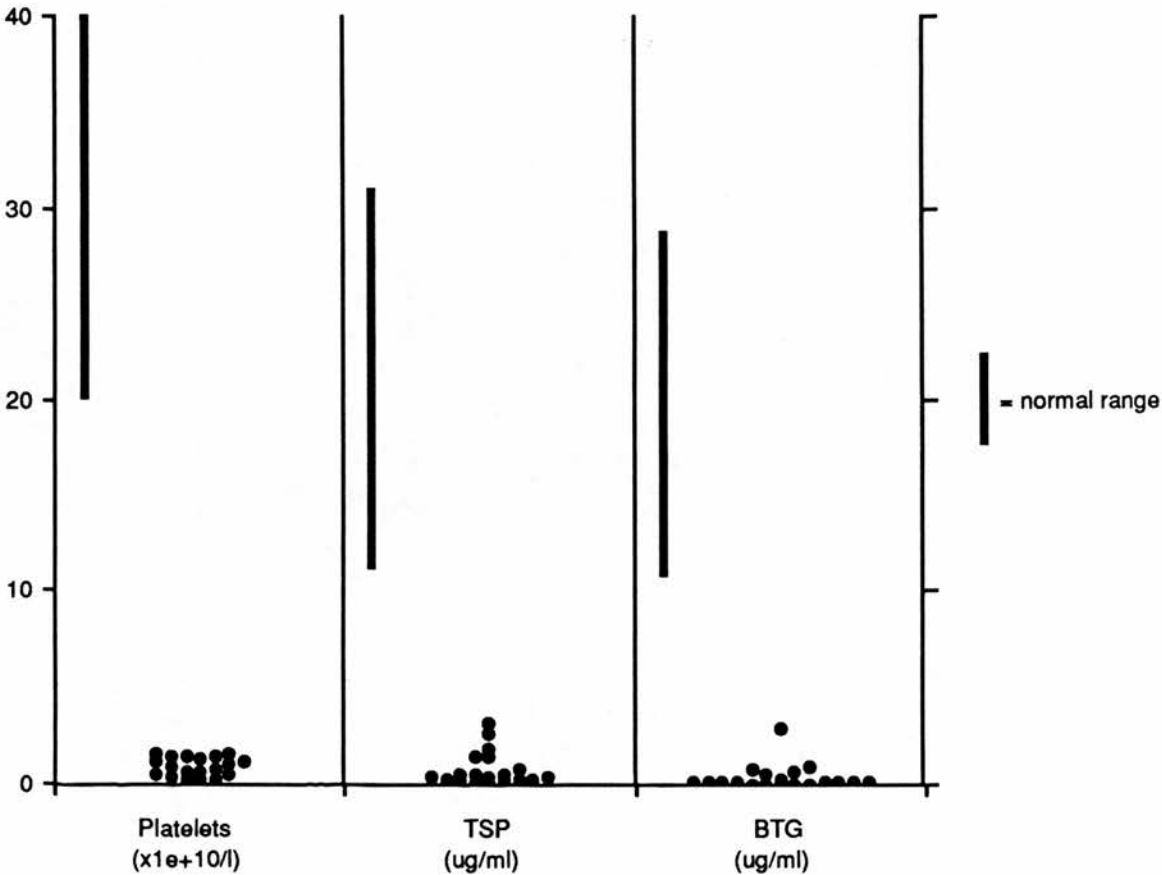
Table 3-4. Plasma samples from thrombocytopenic patients

Patient	Age	Sex	Sample time (days from termination of chemotherapy)
SINGLE SAMPLES			
1	38	M	+ 10
2	16	F	+ 12
3	25	F	+ 9
4	38	M	no chemotherapy
5	46	F	+ 11
6	78	M	no chemotherapy
7	32	F	+ 3
8	63	M	+ 30
9	32	M	+ 19
10	88	F	no chemotherapy
11	79	F	+ 12
12	53	M	+ 18
14	62	F	+ 13
15	57	F	+ 14
16	64	M	+ 24
17	29	M	+ 12
18	74	F	+ 27
19	62	F	+ 7
20	76	M	+ 41
SERIAL SAMPLES			
9	32	M	+ 11 to + 24
13	49	F	- 6 to + 41
14	62	F	0 to + 28

Fig. 3-7a. Platelet count, plasma TSP and BTG in thrombocytopenic patients



g. 3-7b. Platelet count, serum TSP and BTG in thrombocytopenic patients



**Fig. 3-8a. Relationship between plasma TSP and BTG in thrombocytopenic patients**

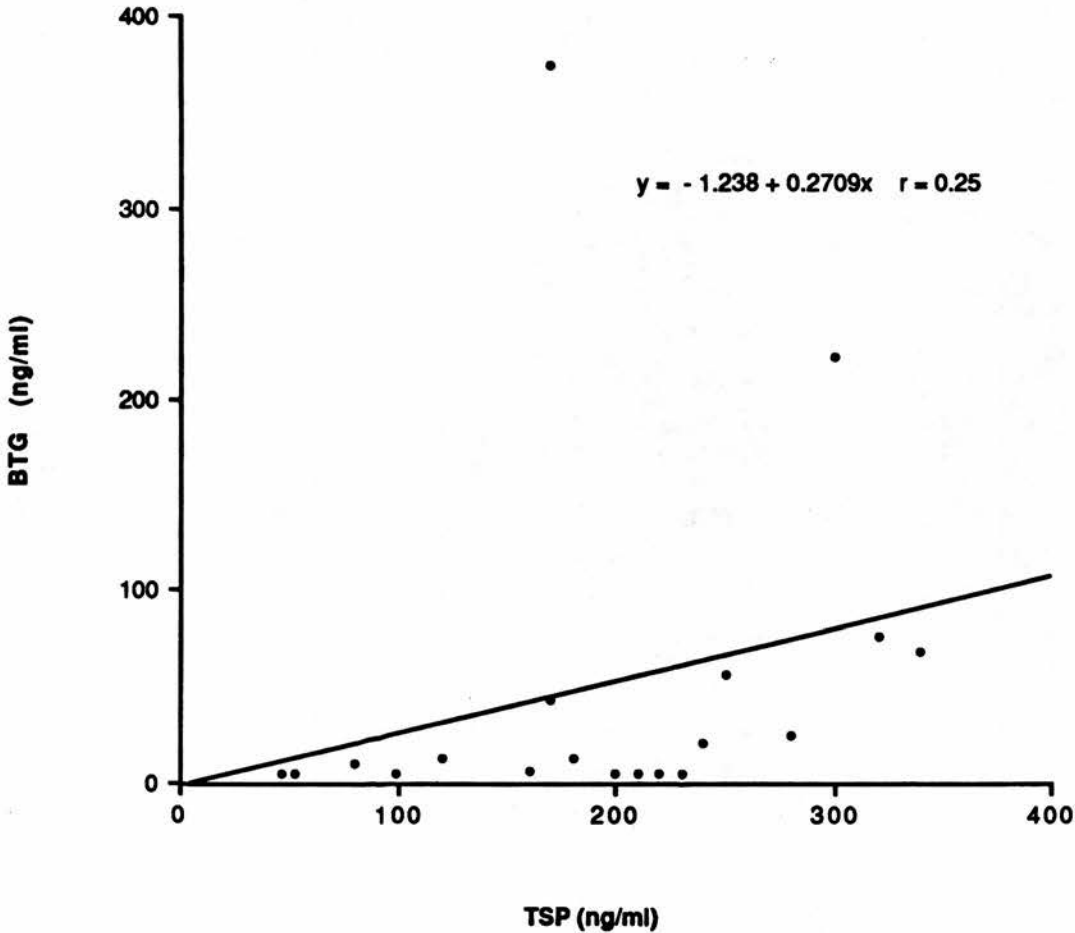
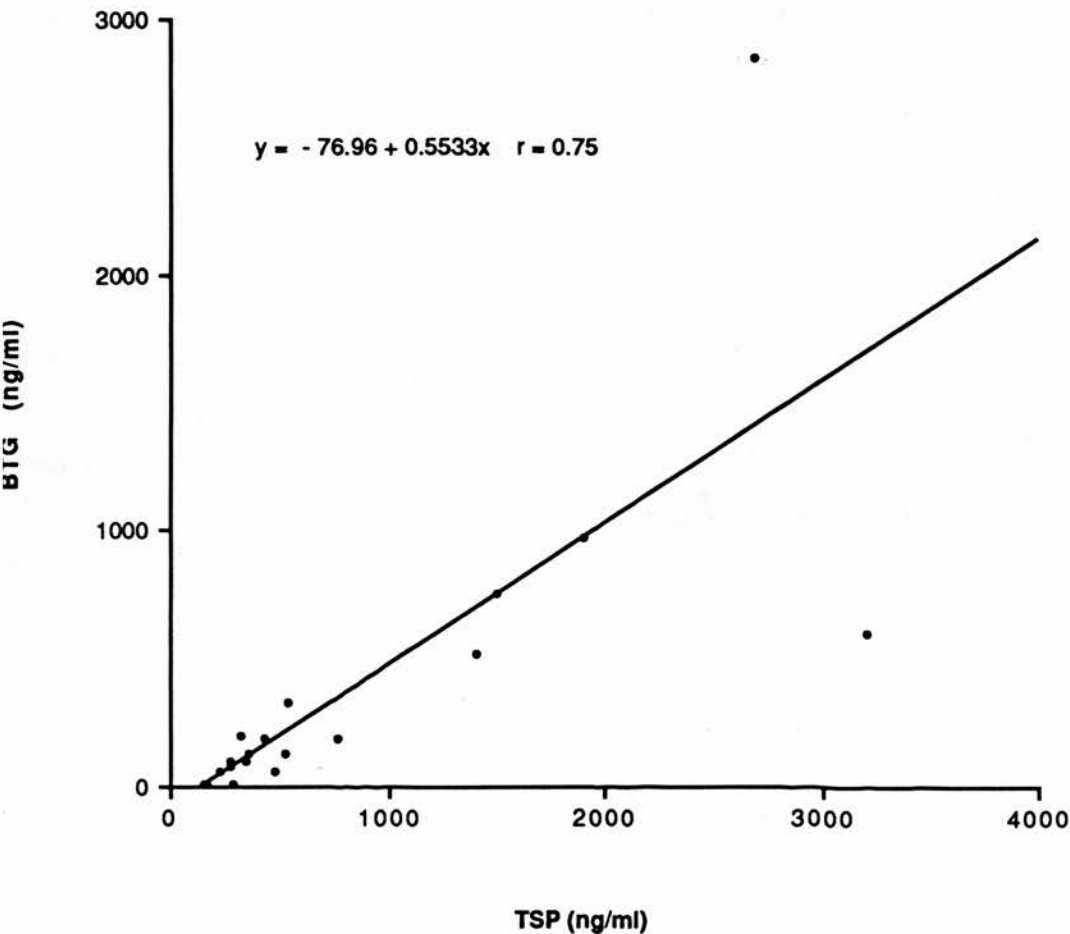


Fig. 3-8b. Relationship between serum TSP and BTG in thrombocytopenic patients



### **3.3.2 Plasma and serum levels of TSP and BTG during recovery from severe bone marrow depression**

Platelet count, plasma and serum levels of TSP and BTG in serial samples taken from three patients during recovery from bone marrow hypoplasia are shown in fig.3-9. Serum concentrations of the two proteins rose and fell in tandem with the platelet count, down to a minimum level which reflected that of plasma.

Similarly, the plasma content of BTG tended to follow the platelet count, though measurement of low values was limited by the sensitivity of the assay (5 ng/ml). While there was some variation in the plasma TSP level which did reflect changes in platelet count, the relationship between the two was not as close as was apparent for BTG. Moreover, the fluctuations in the plasma TSP concentration were superimposed upon a basal level of approximately 50 ng/ml.

### **3.3.3 Relationships between platelet count, plasma and serum levels of TSP and BTG in thrombocytopenic patients**

The relationships between platelet count and the plasma contents of TSP and BTG for all samples from the thrombocytopenic patients are shown in fig.3-10. No statistically discernible correlation was observed between platelet count and plasma TSP ( $r = -0.09$ ;  $P > 0.25$ ), nor between platelet count and plasma BTG ( $r = 0.21$ ;  $P > 0.05$ ).

Fig.3-11 shows the relationships between platelet count and the serum TSP and BTG concentrations. Both TSP ( $r = 0.97$ ;  $P < 0.0005$ ) and BTG ( $r = 0.85$ ;  $P < 0.0005$ ) in serum correlated well with platelet count.

Fig. 3-9a. Relationship between platelet count, plasma TSP and BTG in thrombocytopenic patient 9

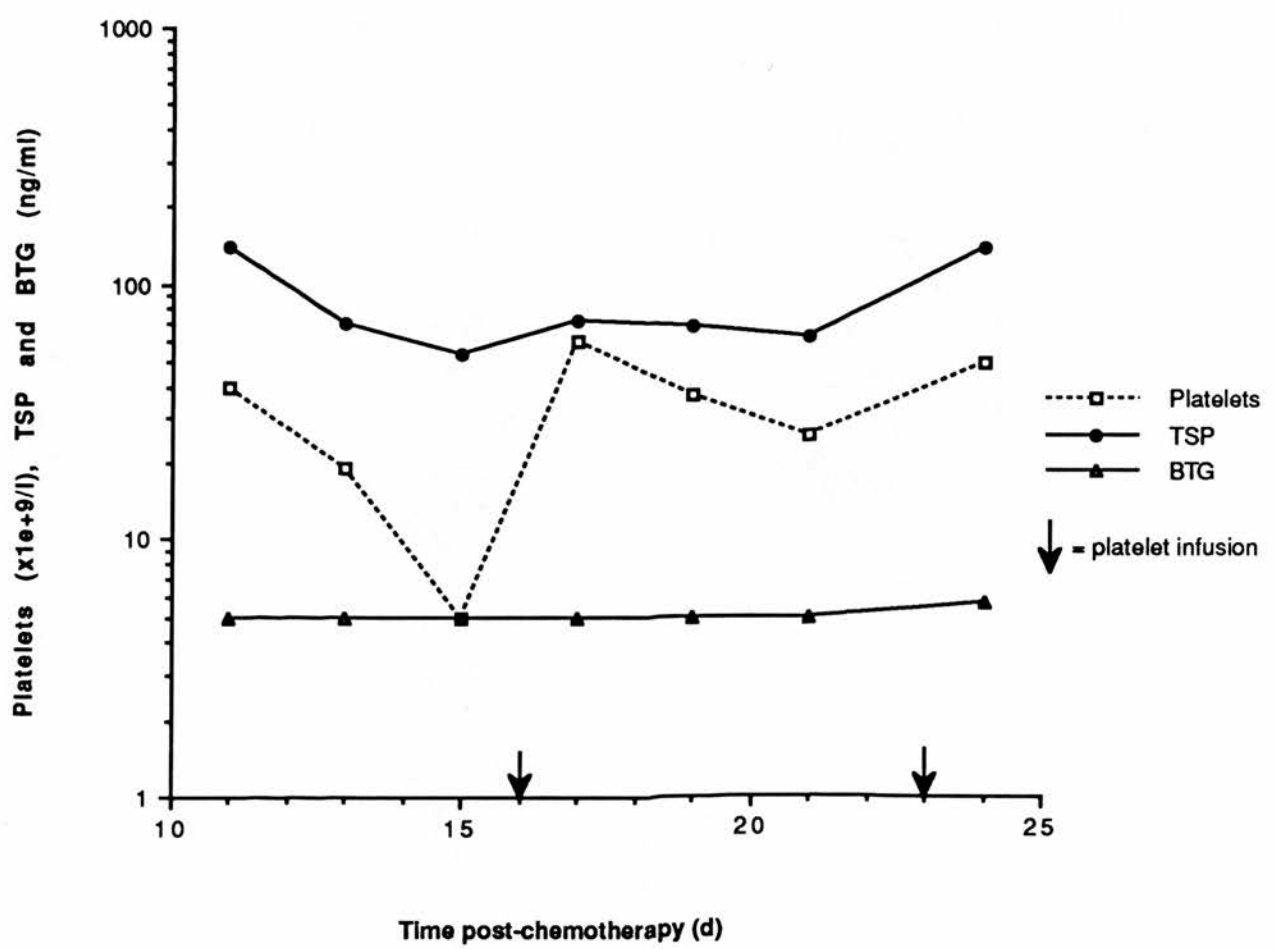




Fig. 3-9b. Relationship between platelet count, serum TSP and BTG in thrombocytopenic patient 9

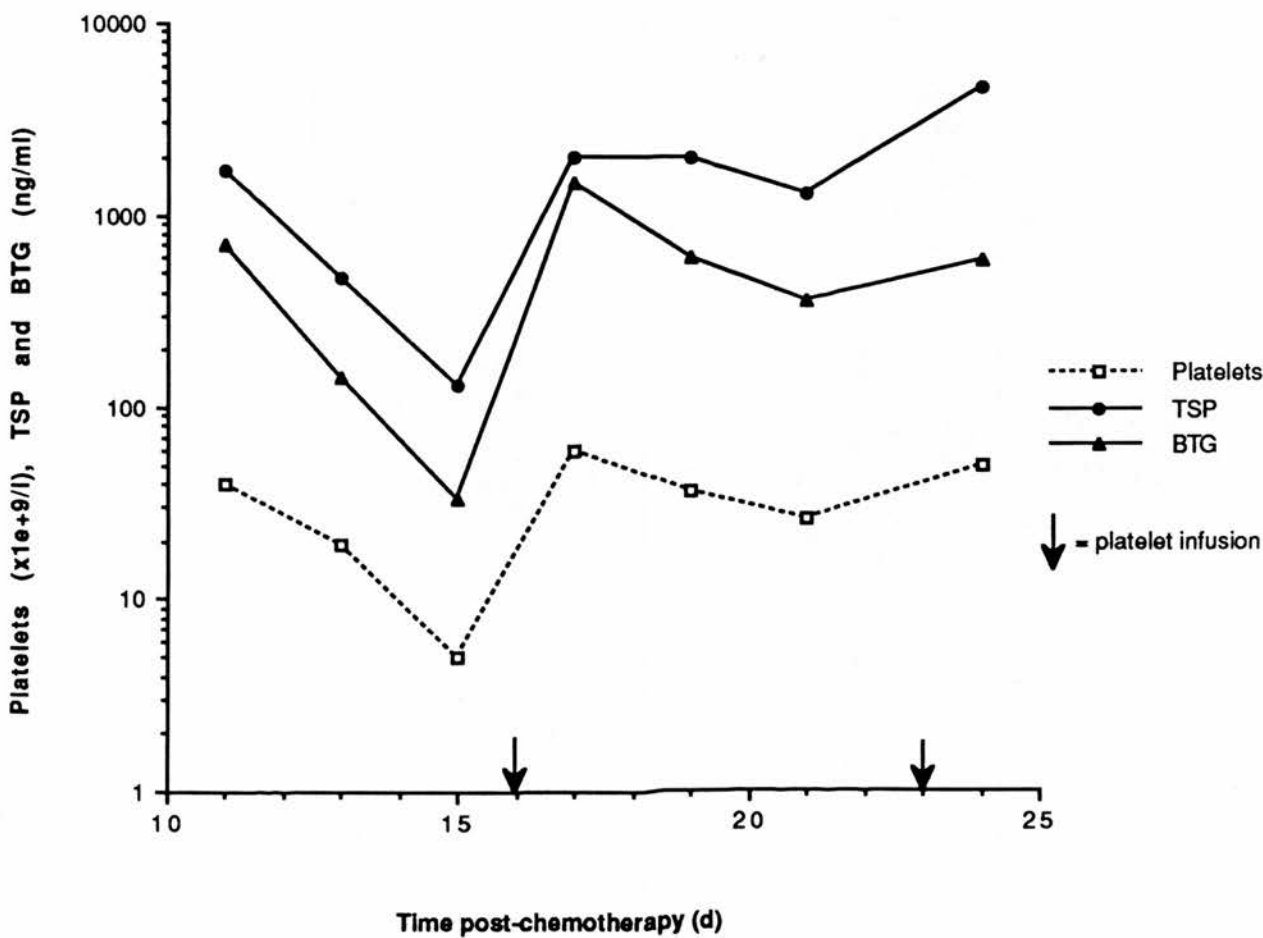
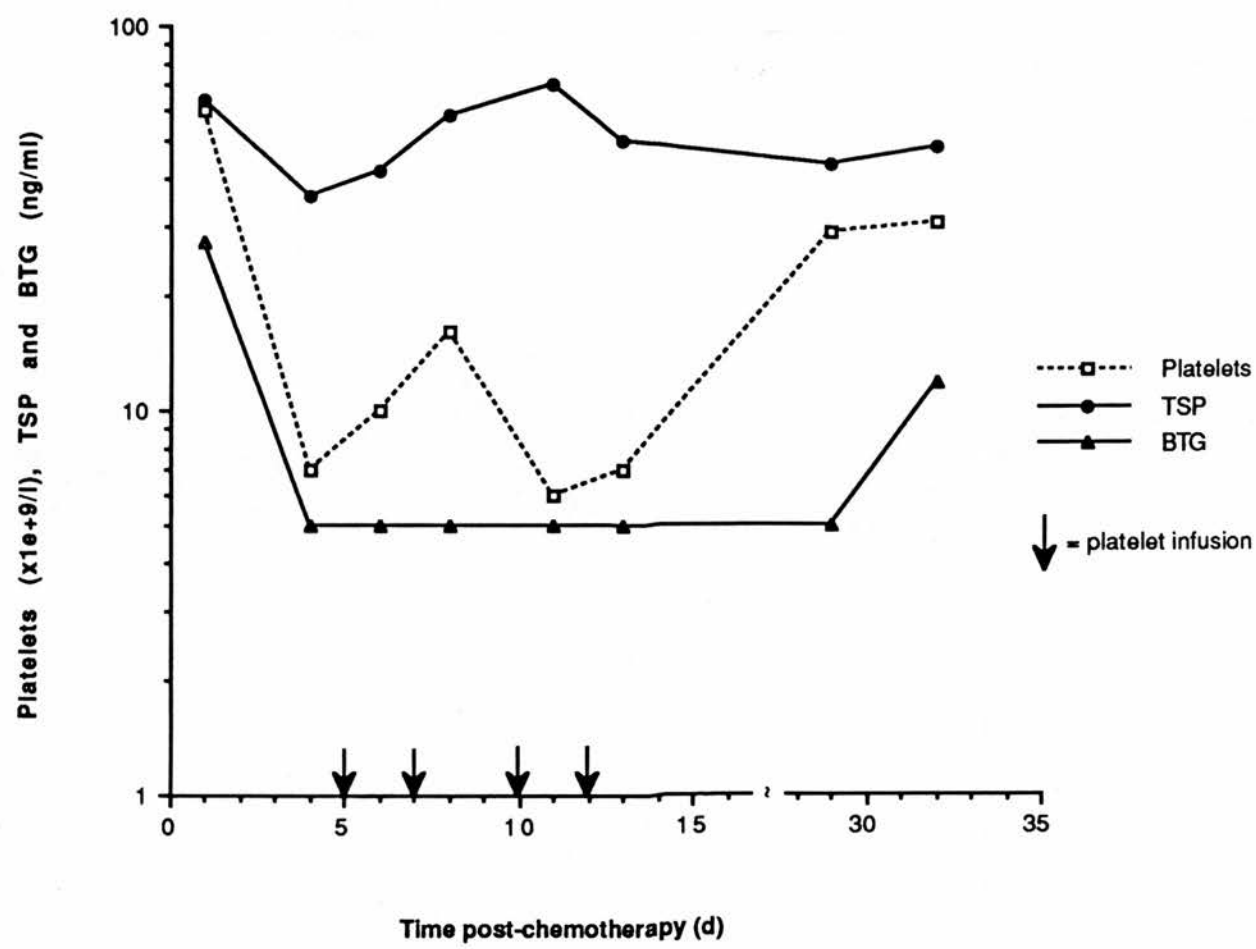


Fig. 3-9c. Relationship between platelet count, plasma TSP and BTG in thrombocytopenic patient 13



**Fig. 3-9d. Relationship between platelet count, serum TSP and BTG in thrombocytopenic patient 13**

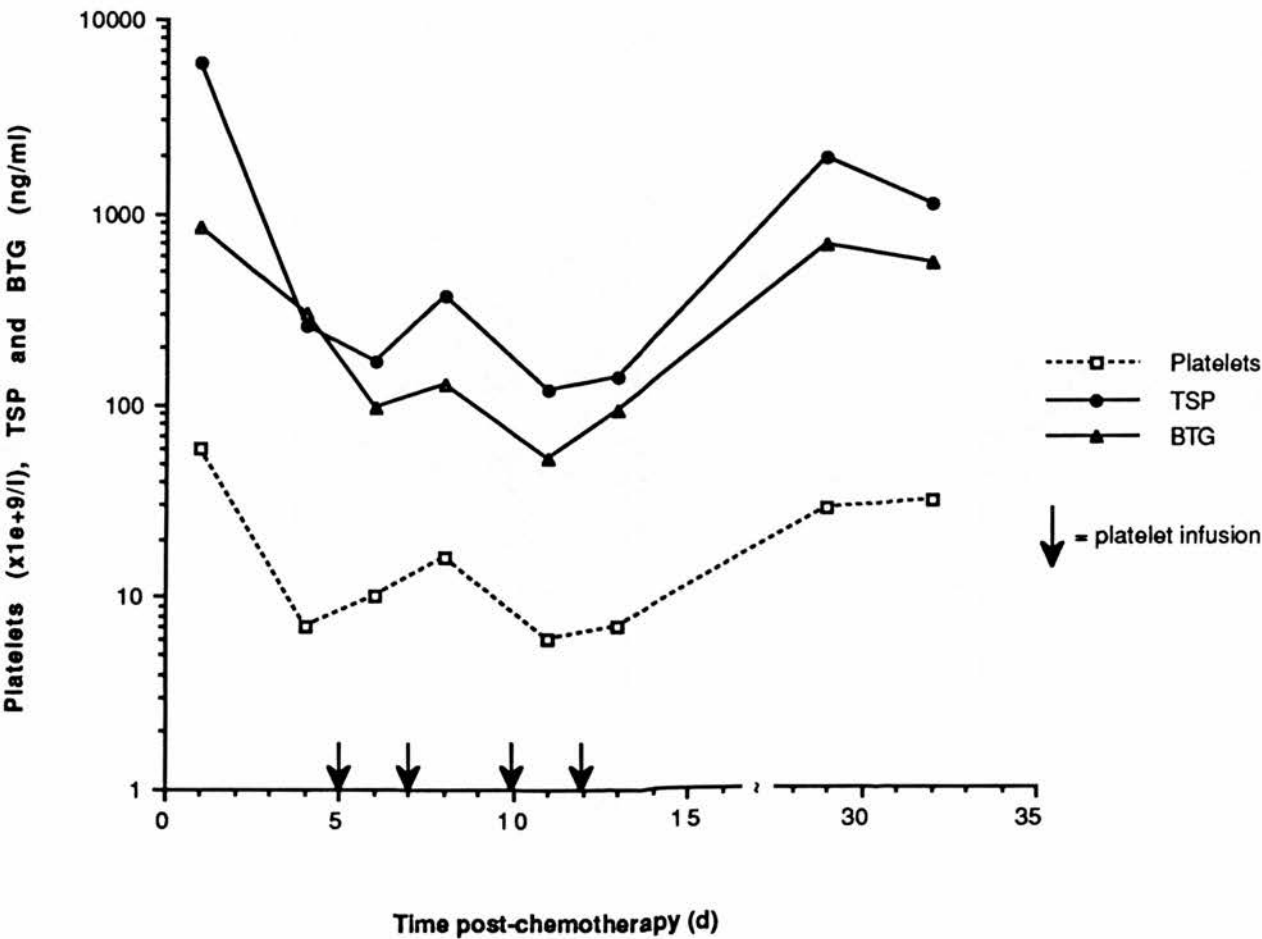


Fig. 3-9e. Relationship between platelet count, plasma TSP and BTG in thrombocytopenic patient 14

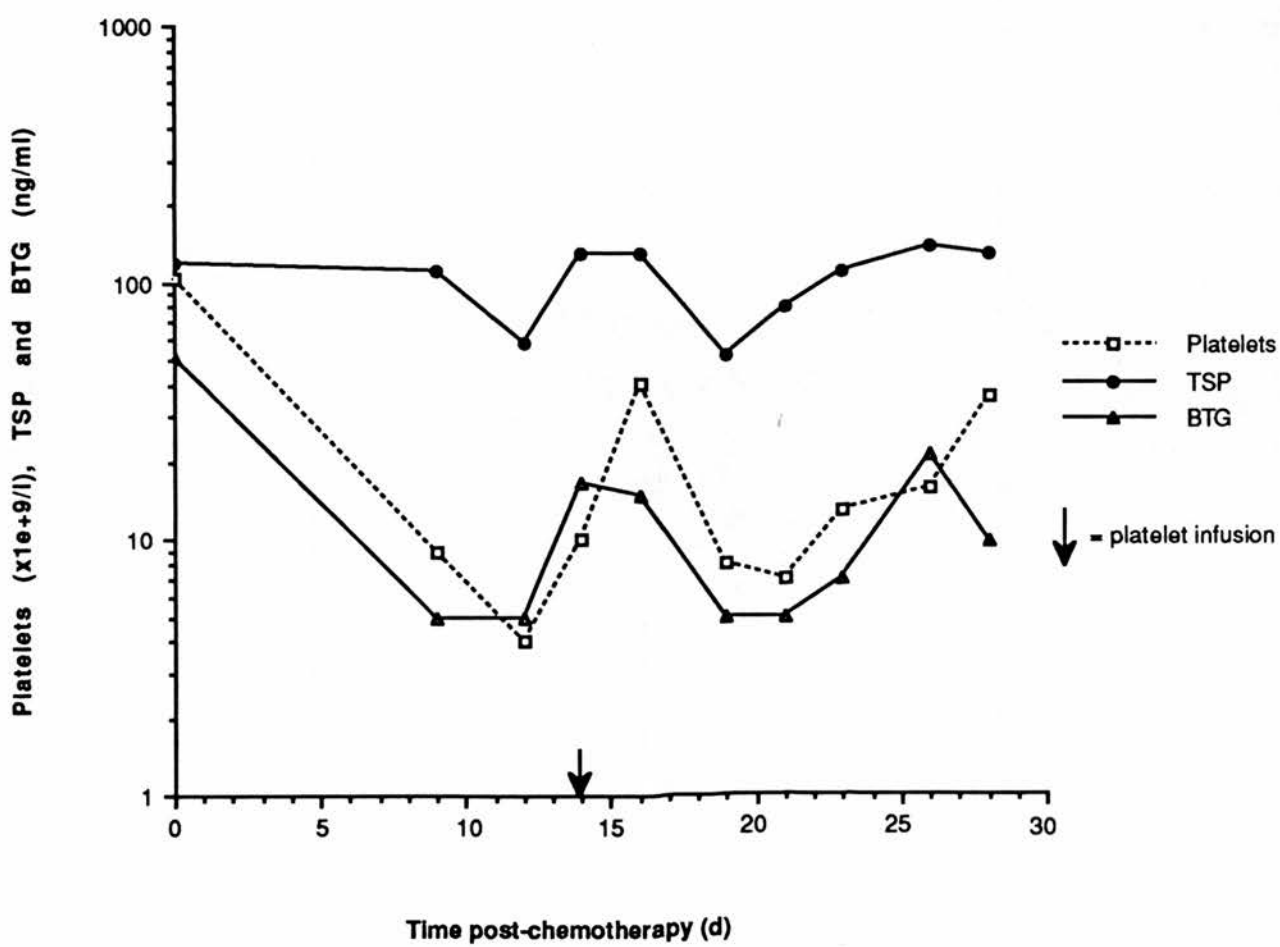
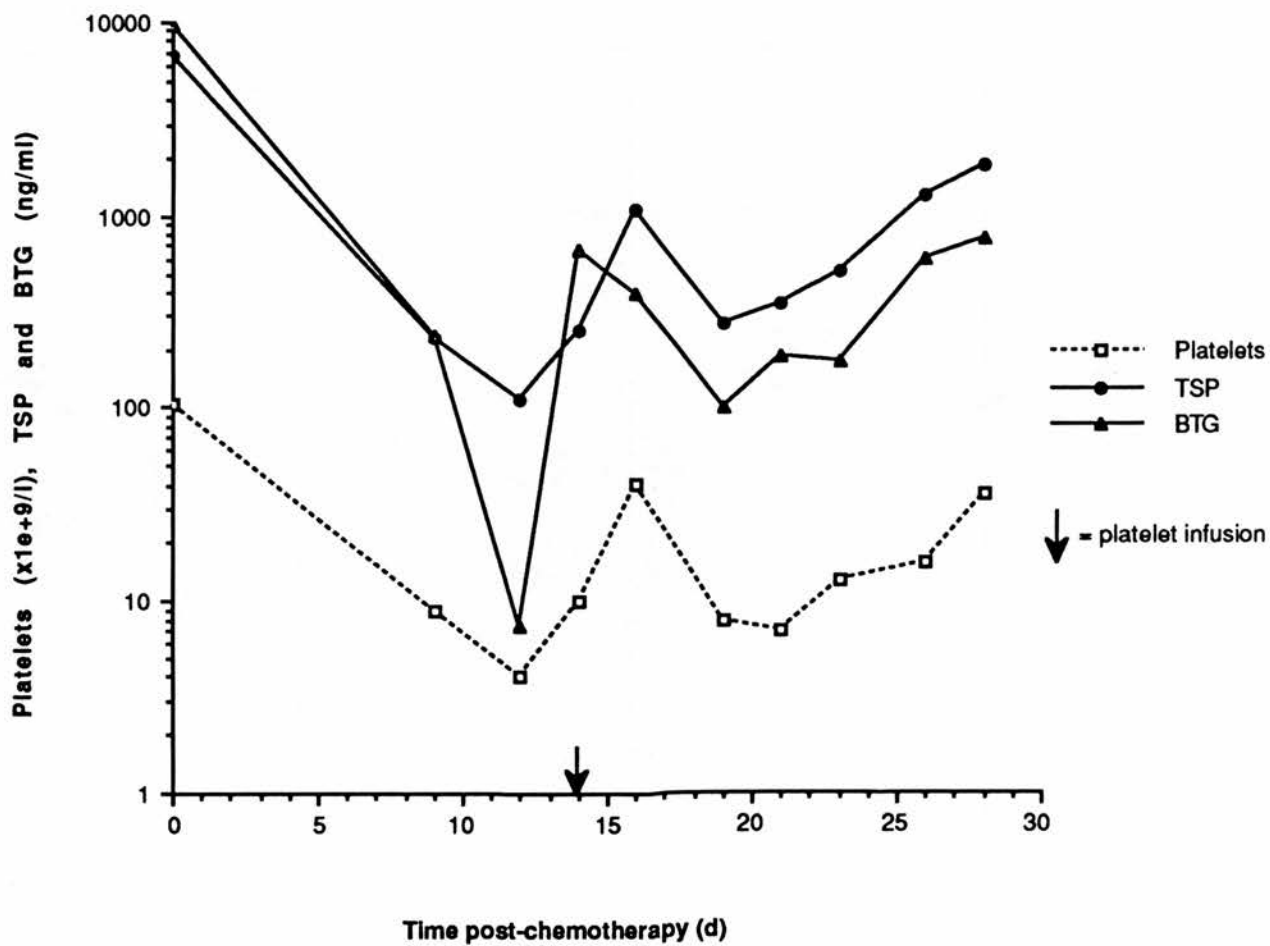
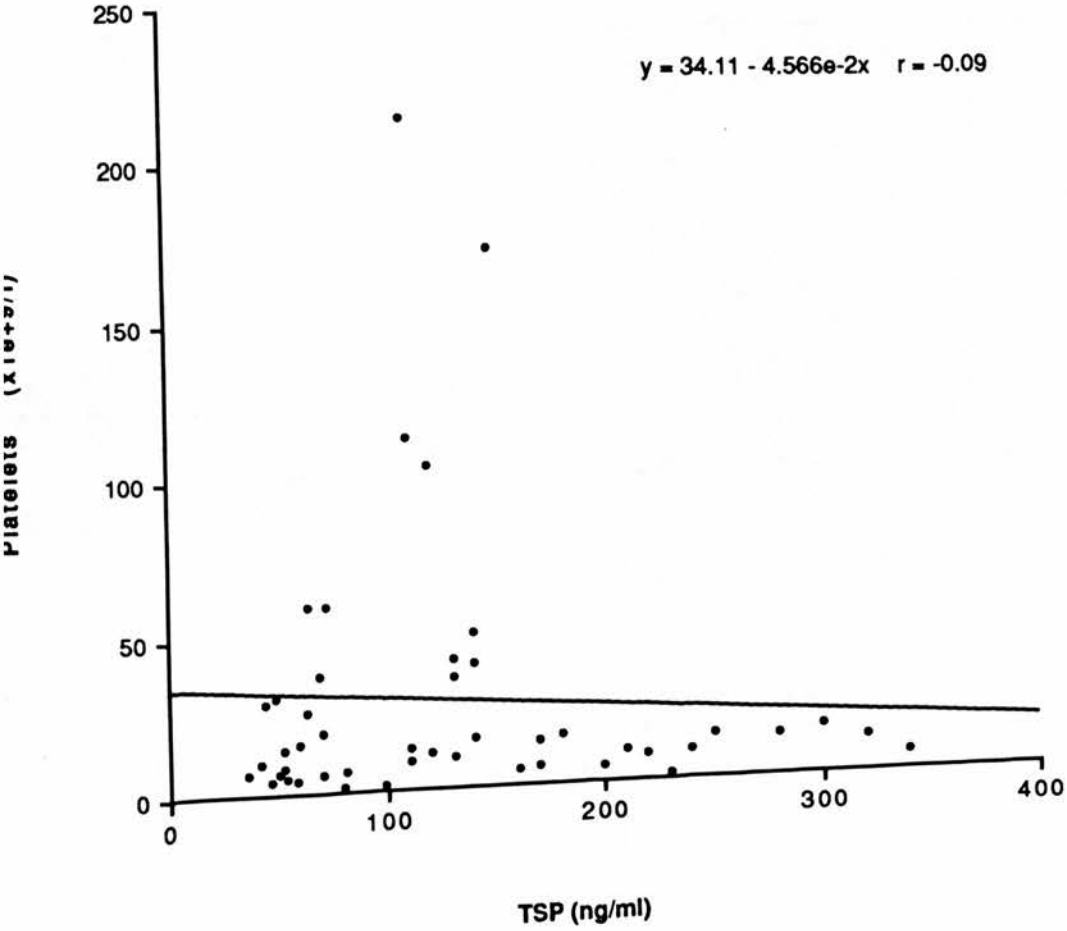


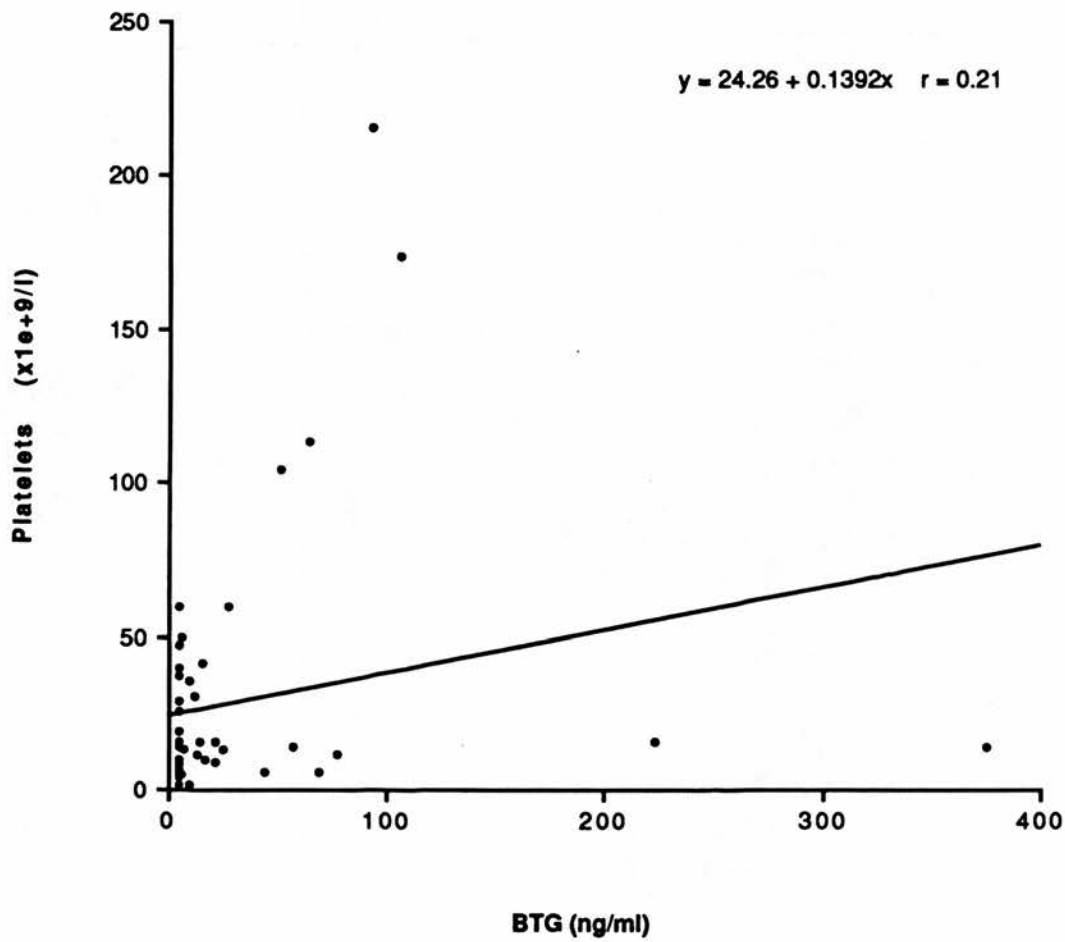
Fig. 3-9f. Relationship between platelet count, serum TSP and BTG in thrombocytopenic patient 14



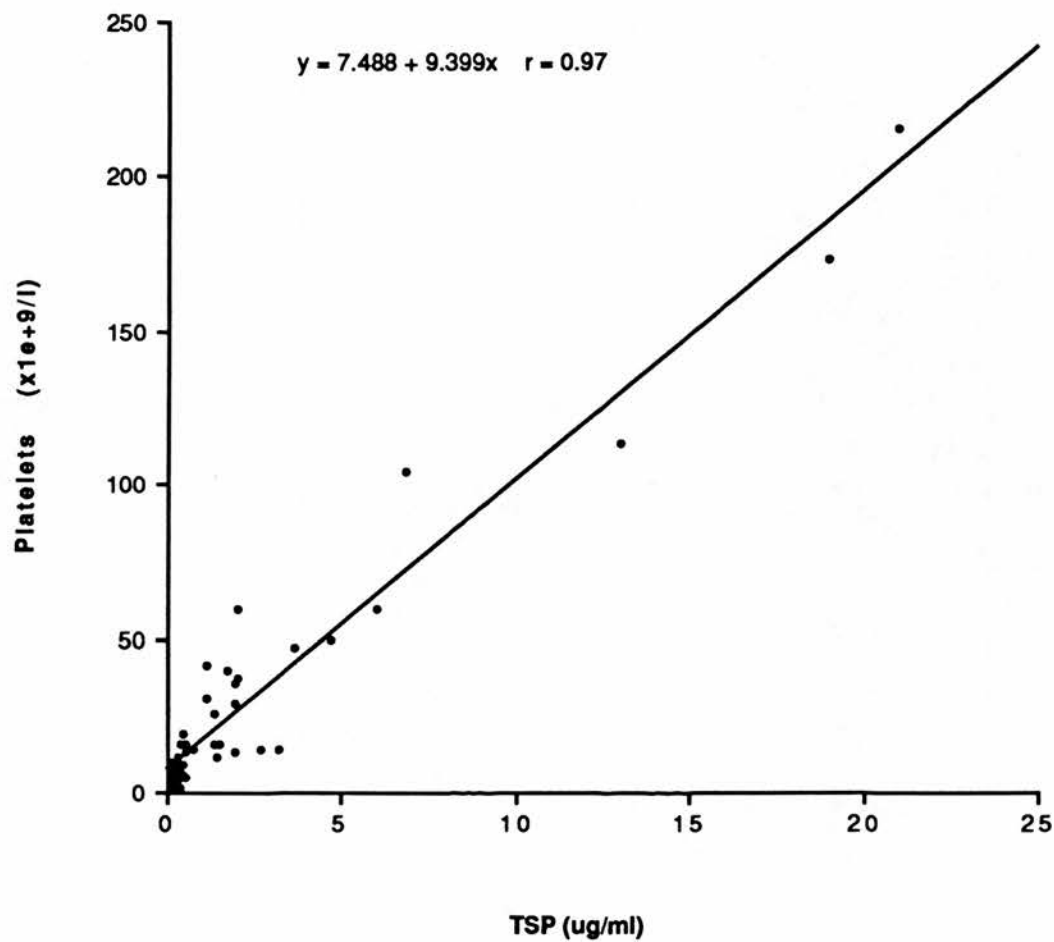
3-10a. Relationship between plasma TSP and platelet count in thrombocytopenic patients



**Fig. 3-1 b. Relationship between plasma BTG and platelet count in thrombocytopenic patients**

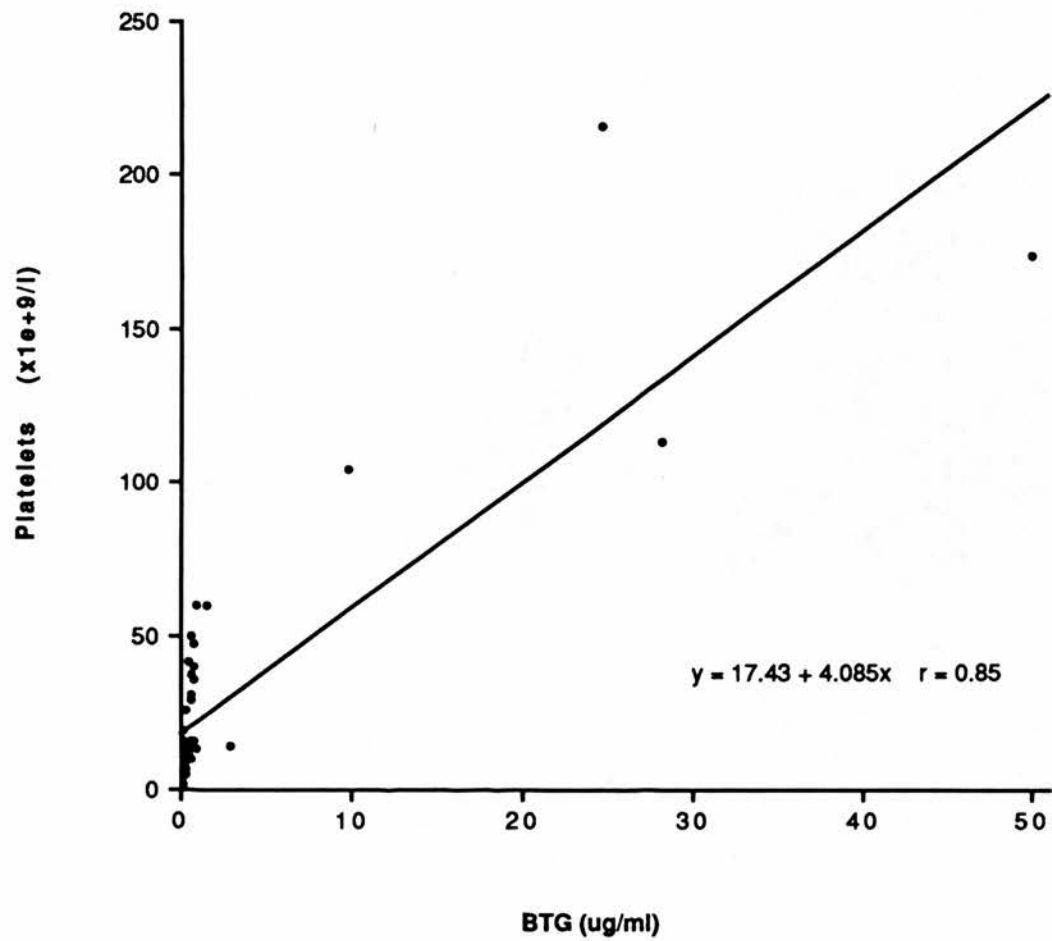


g. 3-11a. Relationship between serum TSP and platelet count in thrombocytopenic patients





g. 3-11b. Relationship between serum BTG and platelet count in thrombocytopenic patients



### **3.4 Plasma TSP in dogs receiving infusions of blood products**

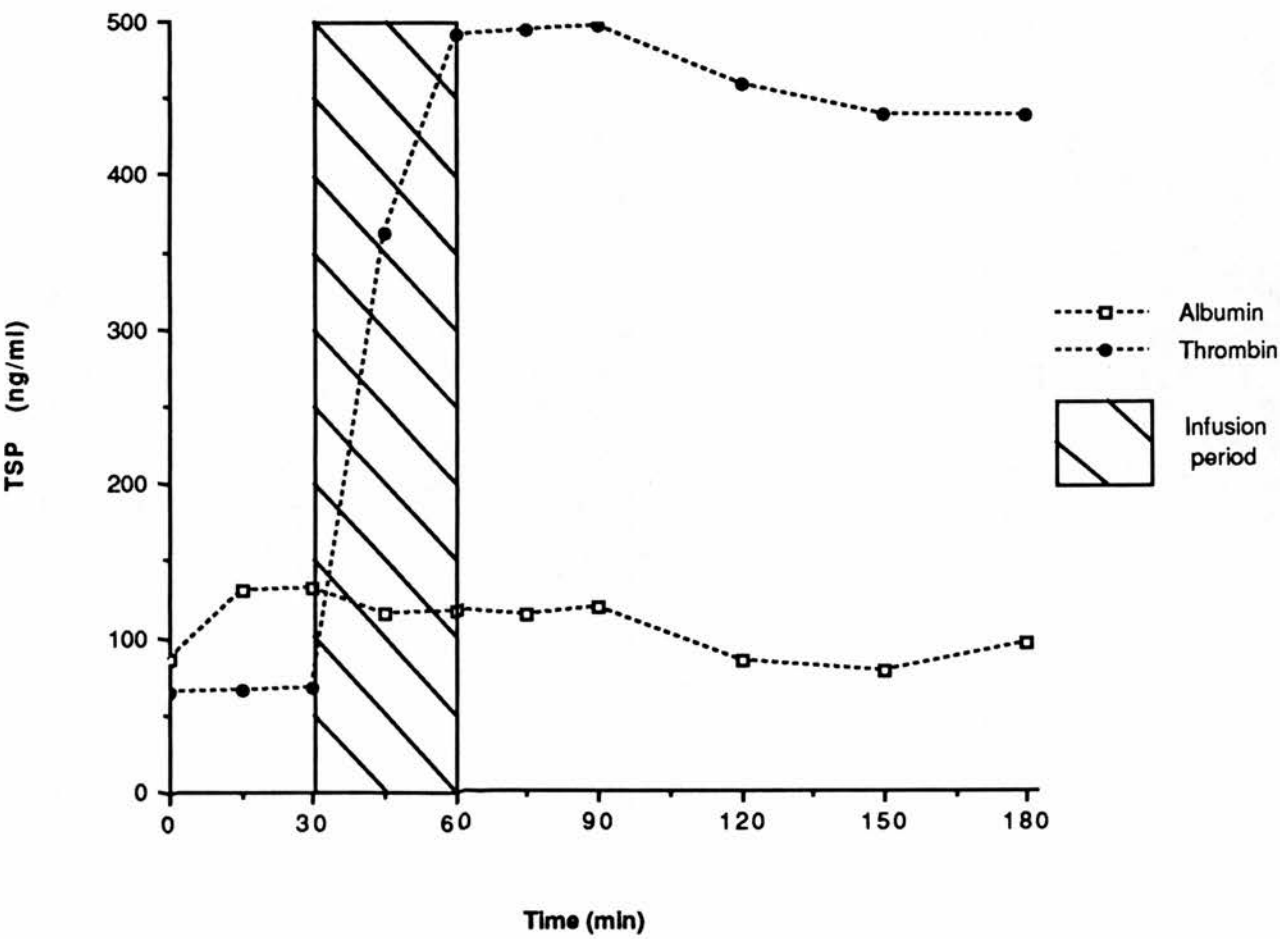
The animal model used to test the thrombogenicity of human blood products has been described by Littlewood et al (1987). Briefly, greyhounds (25 - 30 kg) were anaesthetised and catheterised, isotonic saline was infused via the femoral vein from 0 - 30 min, followed by the test infusion (maximum 140 ml) from 30 - 60 min. Blood samples were taken by separate venepuncture from superficial limb or jugular veins, at 0, 15, 30, 45, 60, 75, 90, 120, 150 and 180 min, into ETP anticoagulant/inhibitor. Test infusions were bovine thrombin (Parke-Davis, New Jersey, USA), human albumin, human factor IX (Scottish National Blood Transfusion Service Protein Fractionation Centre), human factor VII or human factor XI (Churchill Hospital Plasma Fractionation Centre, Oxford).

#### **3.4.1 Infusion of albumin and thrombin**

Plasma levels of TSP in dogs infused with human albumin (250 - 300 mg/kg) or bovine thrombin (150 U/kg) are shown in fig.3-12. Each result represents the mean of six experiments. When albumin was infused, the plasma TSP content remained steady at its normal basal level (approximately 70 ng/ml).

In contrast, infusion of thrombin caused a large and rapid rise in plasma TSP concentration, to approximately 7 times normal at 60 min, which remained at an elevated level for the duration of the experiment. A wide variation in peak plasma TSP concentration was observed between individual animals and ranged from a 3- to a 24-fold increase above the pre-infusion level.

Fig. 3-12. Plasma TSP in dogs infused with albumin or thrombin



#### 3.4.2 Infusion of varying amounts of factor IX

Plasma levels of TSP in dogs infused with human factor IX concentrate (50, 100 or 200 iu/kg) are shown in fig.3-13. Each result represents the mean  $\pm$  SD of three experiments. Infusion of factor IX caused a rapid rise in plasma TSP concentration, which peaked at 60 min, then fell sharply after the infusion was stopped. The peak level of TSP was approximately proportional to the dose of factor IX given. After infusion of 50 iu/kg factor IX, the plasma TSP concentration returned to a normal level by 150 min, but for increasing doses it remained elevated for the duration of the experiment.

#### 3.4.3 Infusion of untreated and heat-treated factor IX

Plasma levels of TSP in dogs infused with a different batch of human factor IX concentrate (100 iu/kg) which had been left untreated, heated for 72 h at 96°C or heated in the presence of antithrombin III (to inhibit generation of thrombin) are shown in fig.3-14. Each result represents the mean of two (heated) or three (unheated, heated + AT III) experiments. Infusion of unheated or heat-treated factor IX produced similar effects on the plasma TSP level. As observed before, the TSP concentration rose rapidly during infusion and fell sharply upon its completion, remaining elevated to the end of the experiment. A similar pattern was obtained for factor IX prepared in the presence of antithrombin III, except that the peak value was reduced, and the TSP concentration had returned to a near-normal level by 180 min.

Fig. 3-13. Plasma TSP in dogs infused with varying amounts of factor IX

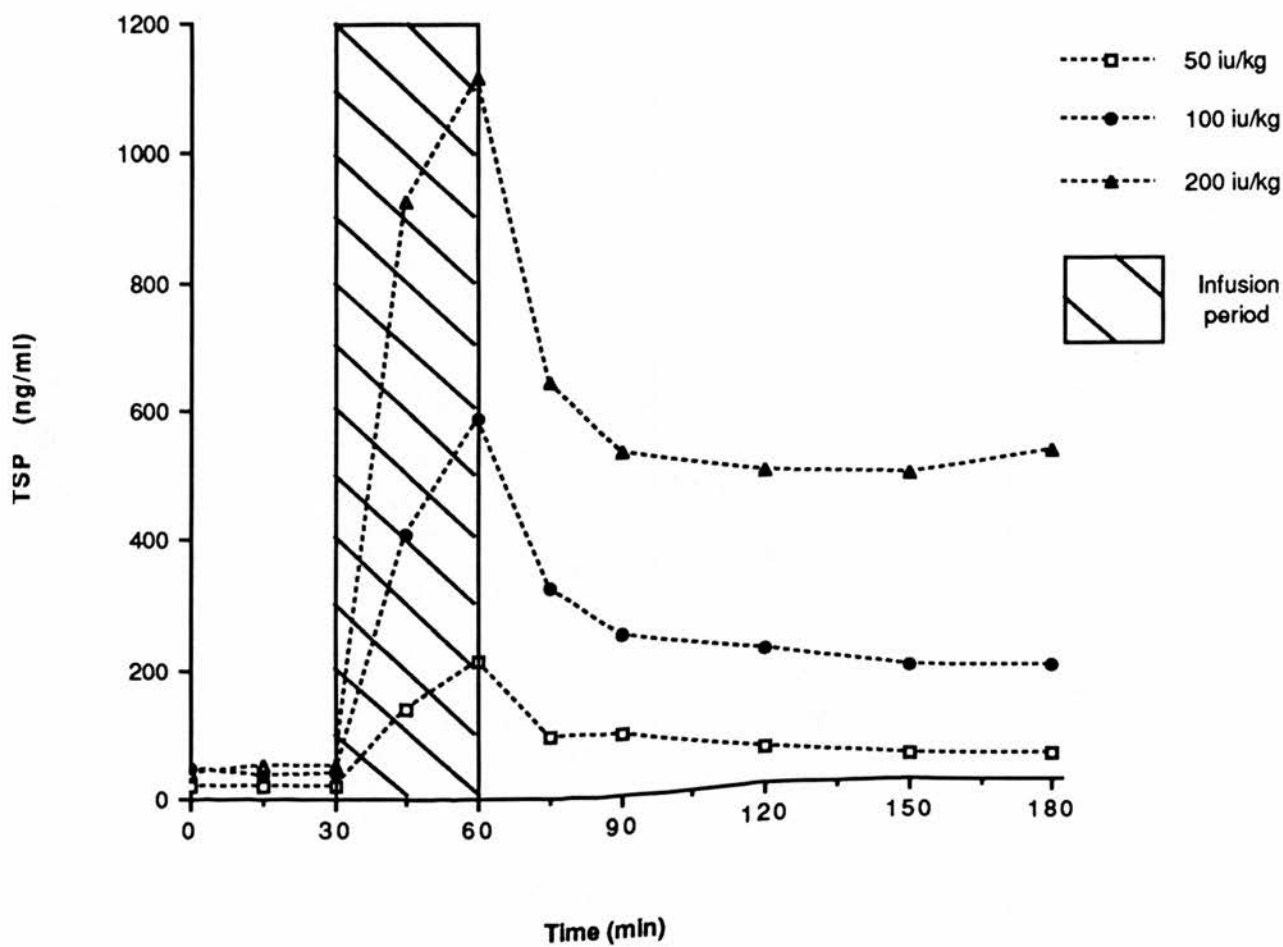
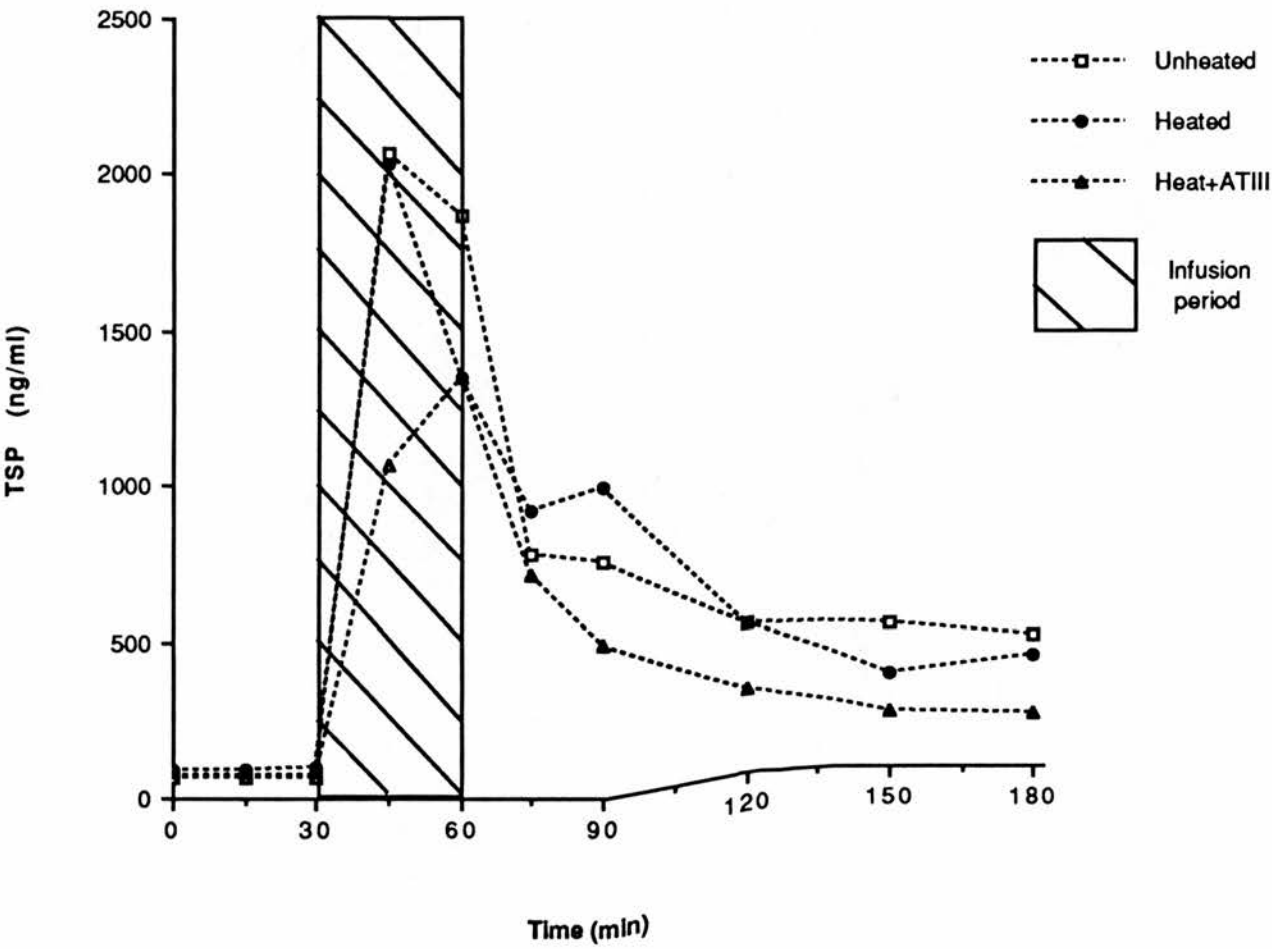


Fig. 3-14. Plasma TSP in dogs infused with factor IX (unheated, heated or heated + ATIII)



#### 3.4.4 Infusion of factor VII and factor XI

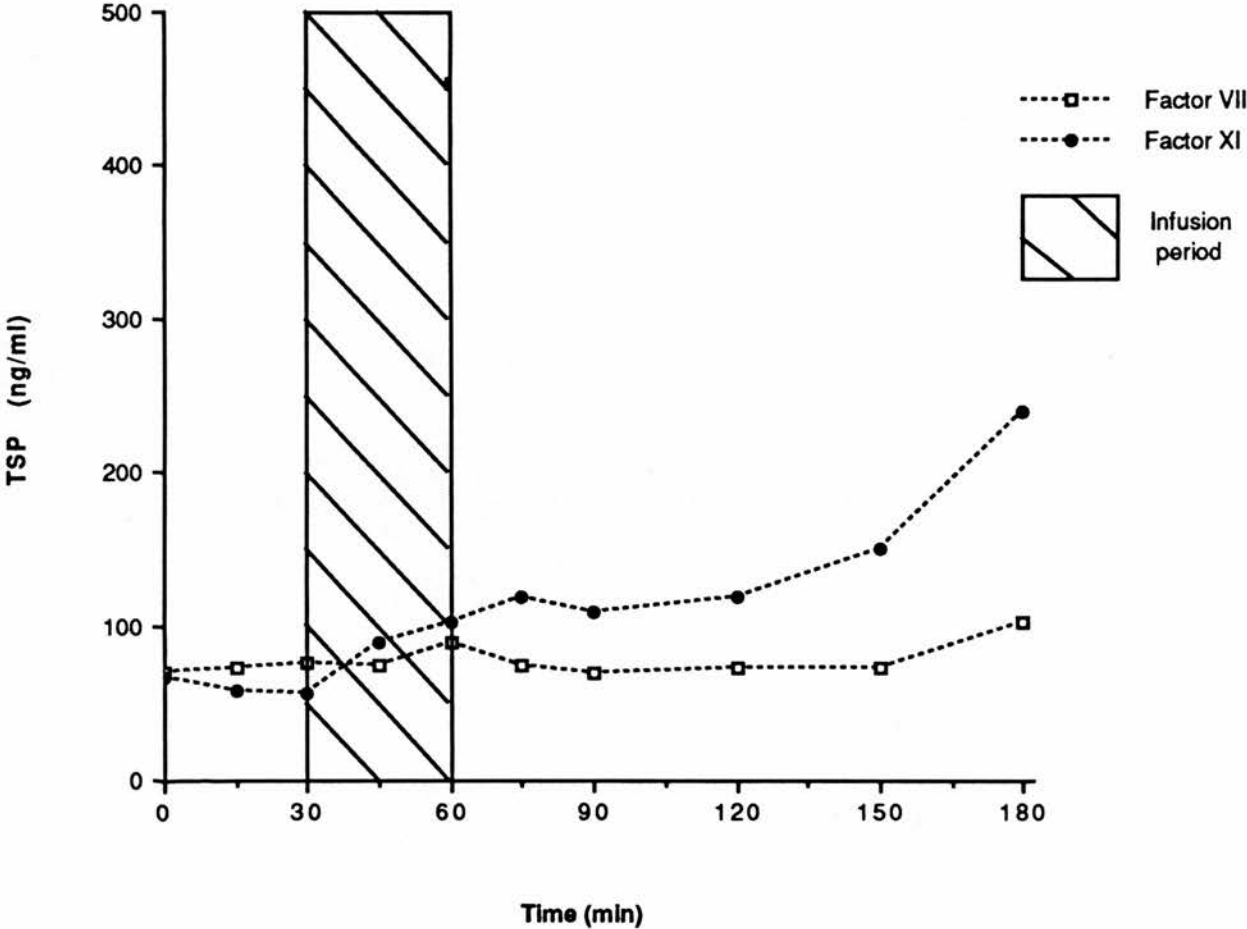
Plasma levels of TSP in dogs infused with human factor VII (heated, 100 iu/kg) or human factor XI (heated, 150 iu/kg) concentrates are shown in fig.3-15. Each result represents the mean of three (factor VII) or two (factor XI) experiments. Infusion of factor VII did not cause any change in the plasma TSP level during the experimental period. Infusion of factor XI resulted in a gradual rise in the TSP content of plasma which continued to the end of the experiment. The increase in TSP concentration became statistically discernible from the control (albumin infusion) after 150 min ( $P < 0.05$ , Wilcoxon-Mann-Whitney test).

#### 3.5 Discussion

Levels of TSP in plasma from normal individuals have been reported by several groups (Saglio & Slayter 1982, Dawes et al 1983, Lane et al 1984, Ffrench et al 1985, Switalska et al 1985, Trzeciak et al 1985, McCrohan et al 1987), showing a relatively large variation (table 3-5). Different assays have varying sensitivities due to the use of different antibodies and methodologies. An important factor is the procedure used for venepuncture and plasma collection; it is vital to prevent in vivo and ex vivo platelet activation which would otherwise falsely elevate the levels of TSP and other  $\alpha$ -granule components. Good sampling technique and the use of an inhibitor cocktail such as ETP (section 2.6) are therefore essential.

Patients undergoing general surgery had similar pre-operative levels of TSP to those in normal plasma, which was expected since there was no evidence of haematological disorders in this group of

Fig. 3-15. Plasma TSP in dogs infused with factor VII or factor XI





patients. However, it was important to demonstrate that the radioimmunoassay, in this operator's hands, produced results which were consistent with those obtained previously and by other groups (table 3-5). Normal plasma BTG concentrations confirmed the absence of excess platelet activation prior to anaesthesia.

Neither general/epidural anaesthesia nor surgery appeared to be thrombogenic, since the plasma levels of TSP and BTG remained steady following surgery. The rise in post-operative plasma vWF concentrations, which was independent of the type of anaesthesia given, could be explained by an increase in endothelial activity involved in the trauma of surgery and the initiation of healing processes by the vessel wall.

The strong positive correlation observed between plasma TSP and BTG in general surgery patients is evidence that the release of platelet  $\alpha$ -granules is a major factor which determines the level of circulating TSP. The lack of correlation between TSP and vWF suggests that different mechanisms influence the plasma content of these glycoproteins. Although endothelial cells synthesise and secrete both TSP and vWF, their intracellular location and control of release are separate (Reinders et al 1985). Therefore activation of the endothelium following surgery could cause an increase in plasma vWF without a concomitant rise in TSP levels.

Studies of Christmas disease patients receiving factor IX infusions revealed that TSP co-purified with factor IX during the production of this plasma fraction. High plasma TSP concentrations found in these patients immediately post-infusion were due to the presence of TSP in factor IX concentrate, and not to any thrombogenicity associated with the infusion material. This was

Table 3-5. Levels of TSP in normal plasma

TSP (mean $\pm$ SD, ng/ml)	N	Anticoagulant	Ref.
163 $\pm$ 103	21	Thrombotect <sup>a</sup>	(1)
226 $\pm$ 100	25	Thrombotect	(1)
132 $\pm$ 72	11	Thrombotect	(1)
107 $\pm$ 71	11	ACD <sup>b</sup>	(1)
209 $\pm$ 141	11	EDTA-K <sub>3</sub>	(1)
105 $\pm$ 31	27	ETP <sup>c</sup>	(2)
211 $\pm$ 31	21	ETP	(3)
264 $\pm$ 53	9	ETP	(3)
54 $\pm$ 16	33	ETP	(4)
61 $\pm$ 11	6	ACD + prostaglandin E <sub>1</sub>	(5)
97 $\pm$ 31	6	ETP	(5)
98 $\pm$ 12	6	ETF <sup>d</sup>	(5)
146 $\pm$ 56	6	EDTA + theophylline	(5)
56 $\pm$ 12	28	ETP	(6)
61 $\pm$ 6	16	EDTA	(7)

<sup>a</sup> EDTA + 2-chloroadenosine + procaine.HCl (Abbott Laboratories)

<sup>b</sup> Acid-citrate-dextrose

<sup>c</sup> EDTA + theophylline + prostaglandin E<sub>1</sub>

<sup>d</sup> EDTA + theophylline + forskolin

#### References:

- 1) Saglio & Slayter 1982
- 2) Dawes et al 1983
- 3) Lane et al 1984
- 4) Ffrench et al 1985
- 5) Switalska et al 1985
- 6) Treciak et al 1985
- 7) McCrohan et al 1987

confirmed by analysis of human factor IX concentrate, of which TSP constituted 0.5% of the total protein. Most of the TSP retained its normal heparin-binding activity, although a minor fraction, which may have been partially degraded or bound to other components in factor IX concentrate, did not.

Clearance of TSP from the plasma of Christmas disease patients occurred in a similar manner to that reported previously for a normal individual (Dawes et al 1983) and in rabbits (Switalska et al 1985). Thus there was rapid initial redistribution followed by a phase of much slower, approximately exponential, loss of TSP from plasma. The routes by which TSP leaves the plasma are not well understood. It is likely that the rapid elimination observed may be due to binding of TSP to platelets and other sites within the vasculature (fibrinogen, fibronectin, plasminogen, HRGP and endothelium) while the slower component could reflect catabolism of this glycoprotein by the liver. Renal clearance is unlikely to be an important route for the catabolism of TSP (Lane et al 1984). In contrast, factor IX was lost from plasma in a much slower, approximately linear manner, such that over 50% of the maximum plasma level achieved following infusion remained after 24 h.

The contribution of platelets to plasma and serum concentrations of TSP was studied in thrombocytopenic patients by relating TSP and BTG levels to their platelet counts. Throughout these studies, the serum content of TSP and BTG correlated closely with the platelet count, and with each other. Since these two glycoproteins are released from platelet  $\alpha$ -granules during clotting, their serum levels simply reflect the number of platelets present.

The relationship between plasma concentrations of the two

glycoproteins and platelet count is, however, more complex. The lack of correlation between plasma BTG and platelet count, and the observation that 42% of individual plasma samples had normal or raised BTG concentrations, despite platelet counts being 10% of normal or less, was unexpected. These results cannot be accounted for by abnormally high platelet BTG contents, since serum levels were those expected from the platelet counts. However, they could reflect release from defective megakaryocytes, which appears to occur in the grey platelet syndrome (Gerrard et al 1980). It should also be emphasised that the kinetics of production and clearance of TSP, BTG and platelets differ and are almost certainly altered in these severely ill patients.

No relationship was demonstrable between plasma TSP concentration and platelet count, nor between the plasma contents of TSP and BTG (in contrast to the case for general surgery patients, where there was a positive correlation between the two). Furthermore, normal levels of TSP were frequently found in plasma in the absence of detectable amounts of BTG, clearly indicating that much of the TSP in plasma can originate from sources other than platelets.

During consecutive sampling of three patients recovering from severe bone marrow depression, the plasma TSP content of both TSP and BTG tended to mirror the platelet count. However, for TSP this pattern was superimposed upon a basal level of approximately 50 ng/ml. The most likely source of this background plasma TSP content, which was independent of the platelet count, is the vascular endothelium.

The response of plasma TSP concentrations in dogs to the

infusion of blood products, can be described in terms of two independent mechanisms which can occur alone or in combination.

Firstly, the sharp rise and fall in TSP levels following factor IX infusion, is due to the presence of human TSP in the infusion material and its rapid clearance from plasma. The animal model gave very similar results to those seen in Christmas disease patients, in that peak levels of TSP following infusion of factor IX concentrate, and the rates of clearance of TSP, were comparable in dogs and humans. Secondly, the prolonged increase in plasma TSP content after thrombin or factor XI infusion, is caused by activation of platelets and release of TSP in the circulation. Although factor IX concentrate was non-thrombogenic at low dose, there was evidence of platelet activation and prolonged release of TSP when larger amounts were infused. Heat treatment of factor IX did not appear to increase its thrombogenicity, but there was some evidence to suggest that addition of antithrombin III to the infusion material could inhibit thrombin generation in the circulation.

## CHAPTER FOUR

### SURFACE-ASSOCIATED THROMBOSPONDIN OF HUMAN PLATELETS

A preliminary study of the surface-associated TSP of human platelets was done in collaboration with Prof N. Crawford and Dr M. Crook, Department of Biochemistry, Royal College of Surgeons of England, London. The feasibility of measuring TSP on formol-fixed platelets in various anticoagulants was investigated, and levels of surface-associated TSP were related to the electrophoretic properties of platelets.

#### 4.1 Comparison of $MgCl_2$ and CPD-adenine-1 anticoagulants

Platelet-associated TSP was measured by radioimmunoassay in suspensions of human platelets prepared by Dr M. Crook, Department of Biochemistry, Royal College of Surgeons of England, London using a method similar to that of George et al (1986). This involved fixation of platelets with formaldehyde (0.4% v/v final concentration) in whole blood anticoagulated with either  $MgCl_2$  (30 mM final concentration) or CPD-adenine-1 (Zuck et al 1977).

When  $MgCl_2$  was used as anticoagulant, the platelet-associated TSP concentration was  $4.29 \pm 2.06$  ng/ $10^6$  cells (mean  $\pm$  SD, n = 7). Assuming that one TSP molecule bound one antibody molecule in the radioimmunoassay, and a molecular weight for TSP of 450 kD, then this was equivalent to 5720 molecules/platelet. However, since TSP is a trimeric glycoprotein, antibody binding may have overestimated

the number of molecules associated with the platelet surface.

Similarly, the platelet-associated TSP in CPD-adenine-1 anticoagulant was  $2.26 \pm 0.42$  ng/ $10^6$  cells, equivalent to 3010 molecules/platelet. The difference between  $MgCl_2$  and CPD-adenine-1 anticoagulants was statistically discernible ( $P < 0.05$ , Student's t test).

#### 4.2 Platelet subpopulations separated by continuous flow electrophoresis

Platelet-associated TSP was studied in surface charge-dependent subpopulations of human platelets separated by continuous flow electrophoresis (Crook and Crawford 1988). Briefly, platelets isolated from  $MgCl_2$ -anticoagulated blood were fixed with formaldehyde (0.4% v/v final concentration) and applied into the cathodal side of the chamber of a Bender & Holbein VAP5 continuous flow electrophoresis apparatus. Platelets were separated into 20 - 25 fractions on the anodal side of the chamber according to increasing electrophoretic mobility. Fractions were assayed for TSP and subdivided into population pools A , B and C of decreasing electronegativity (table 4-1). No statistically discernible difference was observed in the surface TSP of the platelet subpopulations ( $P > 0.05$  for A/B, A/C and B/C comparisons, Student's t test).

Table 4-1. Surface-associated TSP of platelet subpopulations

	Whole pool	Subpopulation		
	'0'	A	B	C
TSP (ng/10 <sup>6</sup> cells)	6.1 ± 1.9	5.8 ± 1.4	5.2 ± 2.6	5.8 ± 1.2

Results expressed as mean ± SD from four experiments.



### 4.3 Discussion

In many clinical situations there is a need for assessment of *in vivo* platelet activation, and although measurements of plasma levels of  $\alpha$ -granule proteins may be useful, they are not always reliable (section 1.7).

Following activation of platelets, TSP is released along with other  $\alpha$ -granule components and may bind to the platelet surface (section 1.6). Dawes et al (1983) estimated that approximately 50% of the TSP secreted by platelets becomes associated with the platelet aggregate during the clotting process. Thus, measurements of surface-associated TSP may provide a more reliable indication of the extent of platelet activation than those of  $\alpha$ -granule proteins in plasma.

Levels of platelet-associated TSP detected in unstimulated, formol-fixed preparations were higher than those reported by George et al (1986). They observed 1763 molecules TSP/platelet when the cells were prepared in 5 mM EDTA, compared with 5720 molecules/platelet in 30 mM  $MgCl_2$  and 3010 molecules/platelet in CPD-adenine-1 for the present study. It is well established that TSP can bind to platelets by a  $Ca^{2+}$ -dependent mechanism (section 1.6) and  $Ca^{2+}$ -chelating anticoagulants, such as EDTA, may cause a proportion of the bound TSP to dissociate from the platelet surface. It has been shown that the use of  $MgCl_2$  as anticoagulant causes no loss of platelet-bound TSP (George et al 1986) and is thought to maintain the surface-associated "halo" of electrostatically- and  $Ca^{2+}$ -linked proteins around the platelet (Crawford et al 1987). This would explain the higher surface-associated TSP concentration found in " $Mg^{2+}$ -platelets"

compared with those in EDTA or CPD-adenine-1 anticoagulant.

Measurement of surface-associated TSP in platelet subpopulations separated by continuous flow electrophoresis showed that the binding of TSP was not dependent on the overall platelet surface charge (electronegativity). The latter has been shown to correlate positively with cell volume, electrophoretic mobility and surface sialic acid, but inversely with membrane -SH group status (Crook & Crawford 1988).

Thus, the surface-associated TSP of human platelets appears to be independent of the size, charge and other intrinsic properties of these cells, but has been shown by George et al (1986) to be increased by thrombin stimulation and in patients with adult respiratory distress syndrome (a clinical condition with increased platelet activation and secretion). It is suggested therefore, that measurement of surface-associated TSP may be a sensitive and specific marker of in vivo platelet activation.

## CHAPTER FIVE

### THROMBOSPONDIN IN BREAST SECRETIONS

The present studies of TSP in breast secretions, and those published previously (Dawes et al 1985, Miller & Dawes 1985, Dawes et al 1987), sought to investigate the source of TSP and the mechanisms controlling its production in the mammary gland. Thus, TSP levels in breast cysts were related to concentrations of other fluid constituents and to the type of lining epithelium, while in human and animal milk the production of TSP was examined during various stages of the reproductive cycle.

Breast cyst fluids were provided by Mr W.N. Scott, University Department of Clinical Surgery, Edinburgh and samples of animal milk by Dr R. Nowak, AFRC Institute of Animal Physiology and Genetics Research, Cambridge.

#### 5.1 Human breast cyst fluids

Platelet-associated proteins have been studied previously in human breast cyst fluids in this laboratory (Miller and Dawes 1985). TSP levels in 50 cyst fluids were 65 - 55000 ng/ml (median = 2500 ng/ml) and 92% were above the normal plasma range. When fluids were classified according to their electrolyte composition, group II ( $\text{Na}^+$ ) cysts ( $\text{Na}^+:\text{K}^+ > 3$ ) had significantly higher levels of TSP than group I ( $\text{K}^+$ ) cysts ( $\text{Na}^+:\text{K}^+ < 3$ ).

#### 5.1.1 Relationship between TSP, EGF and type of cyst fluid

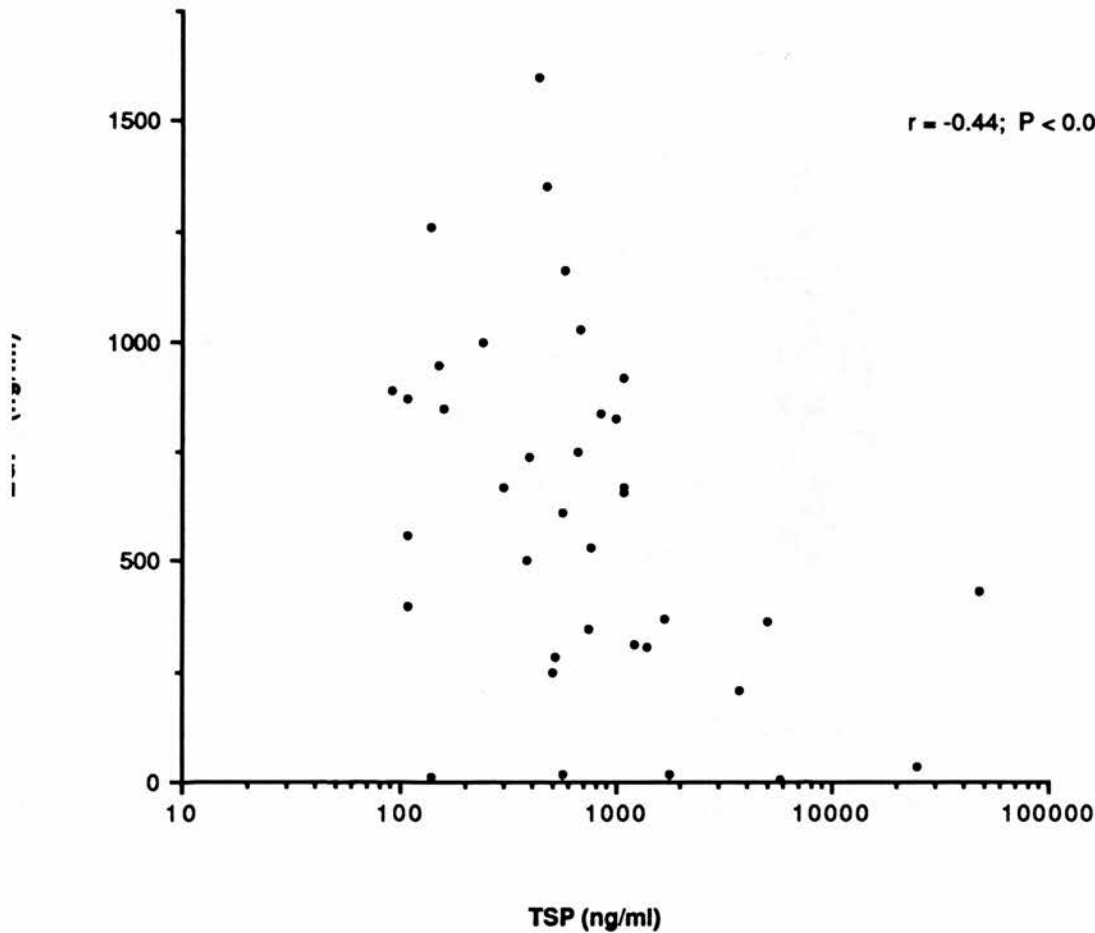
TSP was measured in 37 breast cyst fluids from 31 women, which had been assayed for EGF by radioimmunoassay (section 2.5) and the results published elsewhere (Smith et al 1989). TSP levels were 92 - 48000 ng/ml (median = 580 ng/ml) and 29/37 (78%) were above the normal plasma range. A negative correlation which was statistically discernible was observed between TSP and EGF in breast cyst fluids ( $r_s = -0.44$ ,  $P < 0.005$ , Spearman rank correlation test) (fig.5-1).

When the fluids were classified as above, group II ( $\text{Na}^+$ ) cysts had a higher TSP content ( $n = 15$ , median = 1100 ng/ml, range = 140 - 5800 ng/ml) than group I ( $\text{K}^+$ ) cysts ( $n = 20$ , median = 385, range = 92 - 48000) and the difference was statistically discernible ( $P < 0.005$ , Wilcoxon-Mann-Whitney test) (fig.5-2). Conversely, the EGF concentration in group I ( $\text{K}^+$ ) fluids ( $n = 20$ , median = 799.5 ng/ml, range = 307.6 - 1599.0 ng/ml) was higher than that in group II fluids ( $n = 15$ , median = 312.6, range = 7.9 - 1158.9) and the difference was also statistically discernible ( $P < 0.001$ ) (fig.5-3).

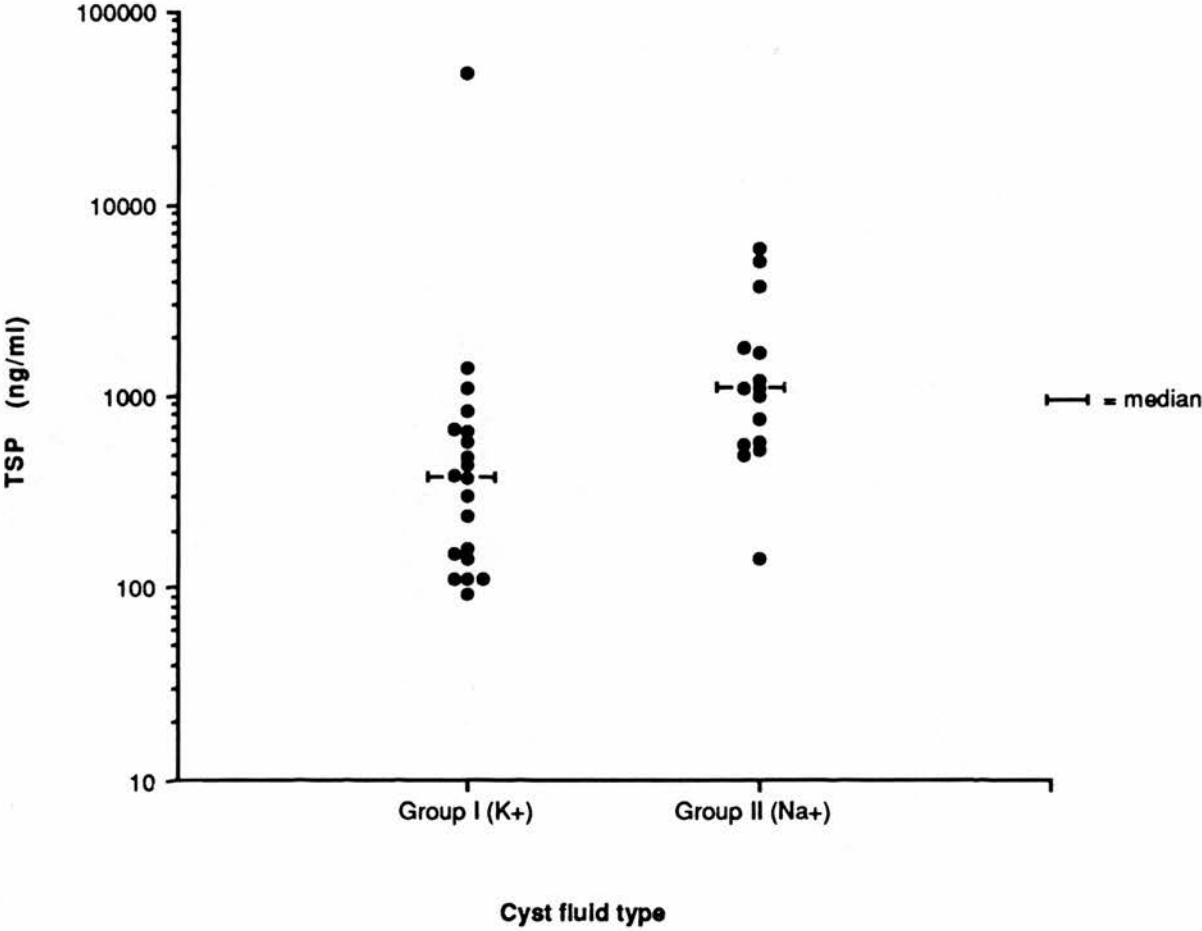
#### 5.1.2 Analysis of cyst fluid by heparin-Sepharose affinity chromatography

A single group II ( $\text{Na}^+$ ) cyst fluid (volume = 13 ml, TSP content = 2800 ng/ml) was centrifuged at 40000 g (18000 rpm) in a Sorvall RC-5B centrifuge with type HS-4 rotor (DuPont Instruments) for 60 min at 4°C in the presence of the protease inhibitors PMSF (100 mM) and leupeptin (6 mM). The supernatant was filtered (AP20 prefilter and GS 0.22  $\mu\text{m}$  membrane filter; Millipore) and 5 ml was applied to a heparin-Sepharose CL-6B column (2 x 24 cm, Pharmacia)

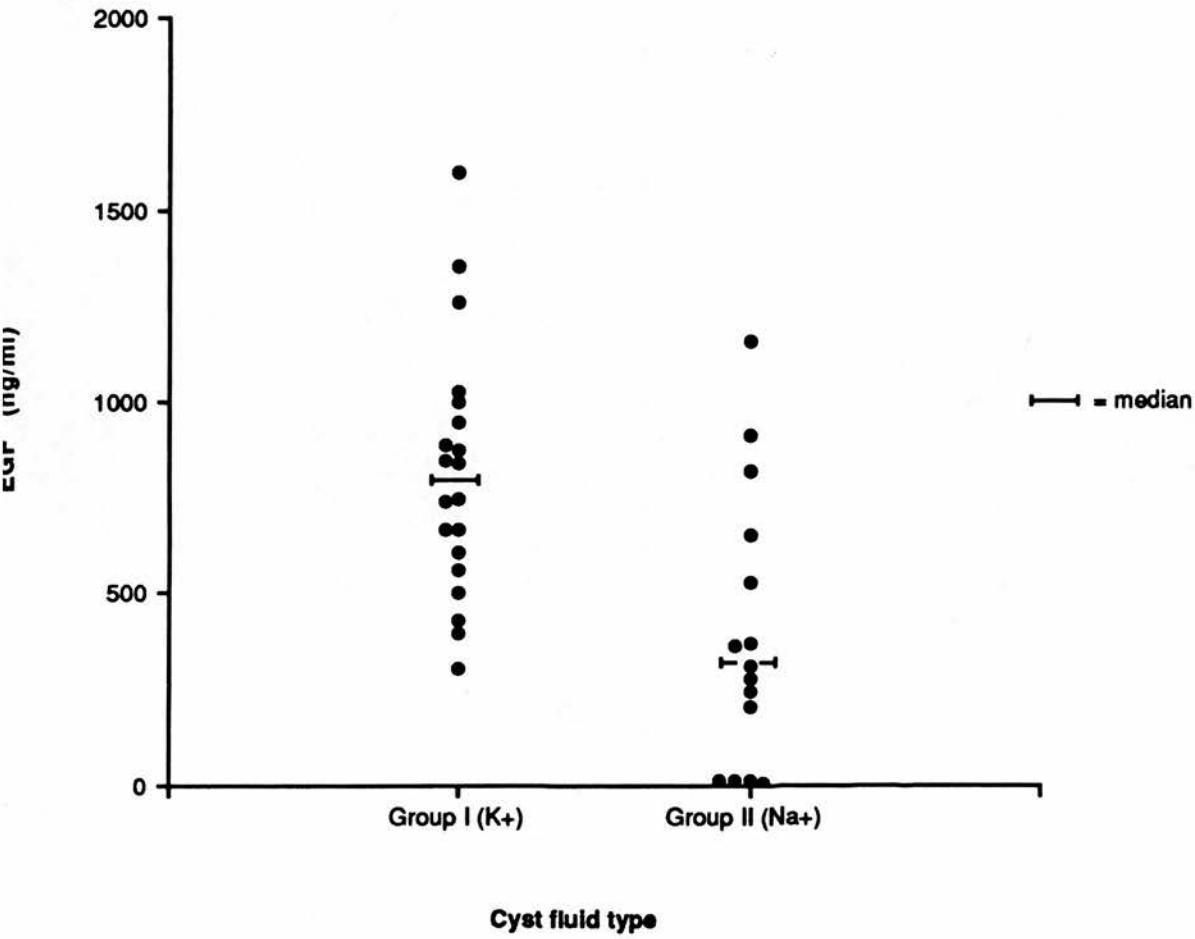
**Fig. 5-1. Relationship between TSP and EGF in human breast cyst fluids**



**Fig. 5-2. TSP in human breast cyst fluids classified according to their electrolyte composition**



**Fig. 5-3. EGF in human breast cyst fluids classified according to their electrolyte composition**



equilibrated in 15 mM Tris-HCl, 0.15 M NaCl, 0.02% NaN<sub>3</sub>, pH 7.4, at a flow rate of 0.4 ml/min. The column was eluted stepwise with buffers of increasing NaCl concentration, and fractions (4 ml) were collected. Each fraction was measured for protein (OD<sub>280</sub>) and TSP content, and the elution profile is shown in fig.5-4.

All of the protein in cyst fluid which was detectable by absorbance measurement at 280 nm was eluted by 0.15 M NaCl. The TSP-like antigenicity of cyst fluid was separated into three peaks eluted by 0.15 M, 0.25 M and 0.55 M NaCl.

## 5.2 TSP in milk

It was previously shown that TSP was a component of the aqueous phase of bovine milk, that the gel filtered protein isolated from goat colostrum had a major peak at 450 kdal together with fragments of low molecular weight, and that TSP from goat colostrum displayed a different proteolytic fragmentation pattern compared with TSP isolated from human platelets, endothelial cells and fibroblasts (Dawes et al 1987).

### 5.2.1 Cow and goat milk

Fresh animal milk samples were stored at -20°C until assayed for TSP and total protein. Daily milk samples were taken from two cows and one goat during their normal oestrous cycles. There was considerable variation in milk TSP content, both within and between animals. No pattern in TSP or total protein levels was observed which corresponded with the oestrous cycle, as shown by the example in fig.5-5.

Milk samples were also taken on successive days from a goat



Fig. 5-4. Heparin-Sepharose affinity chromatogram of a group II breast cyst fluid

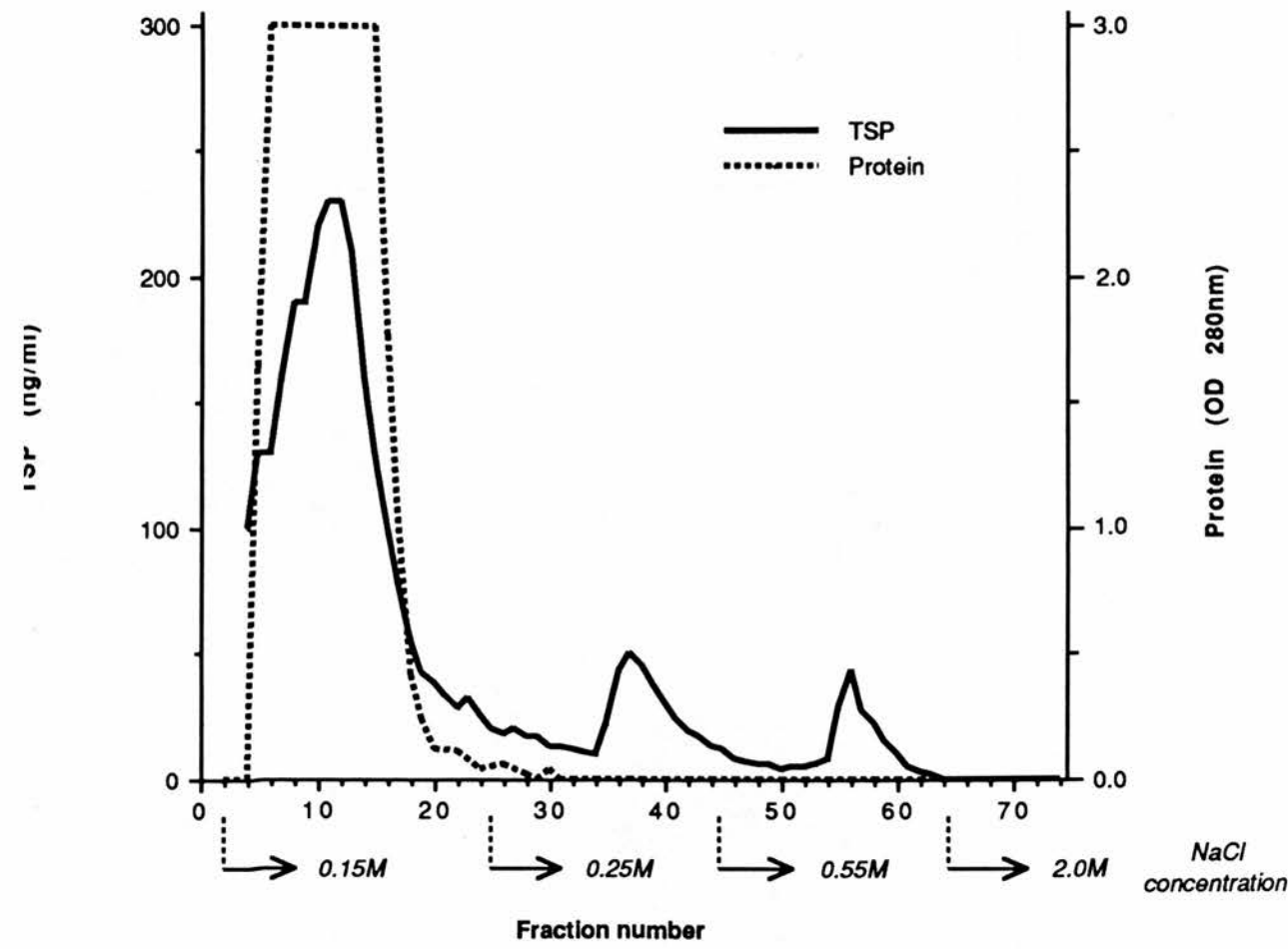


Fig. 5-5. Daily levels of TSP and protein in bovine milk

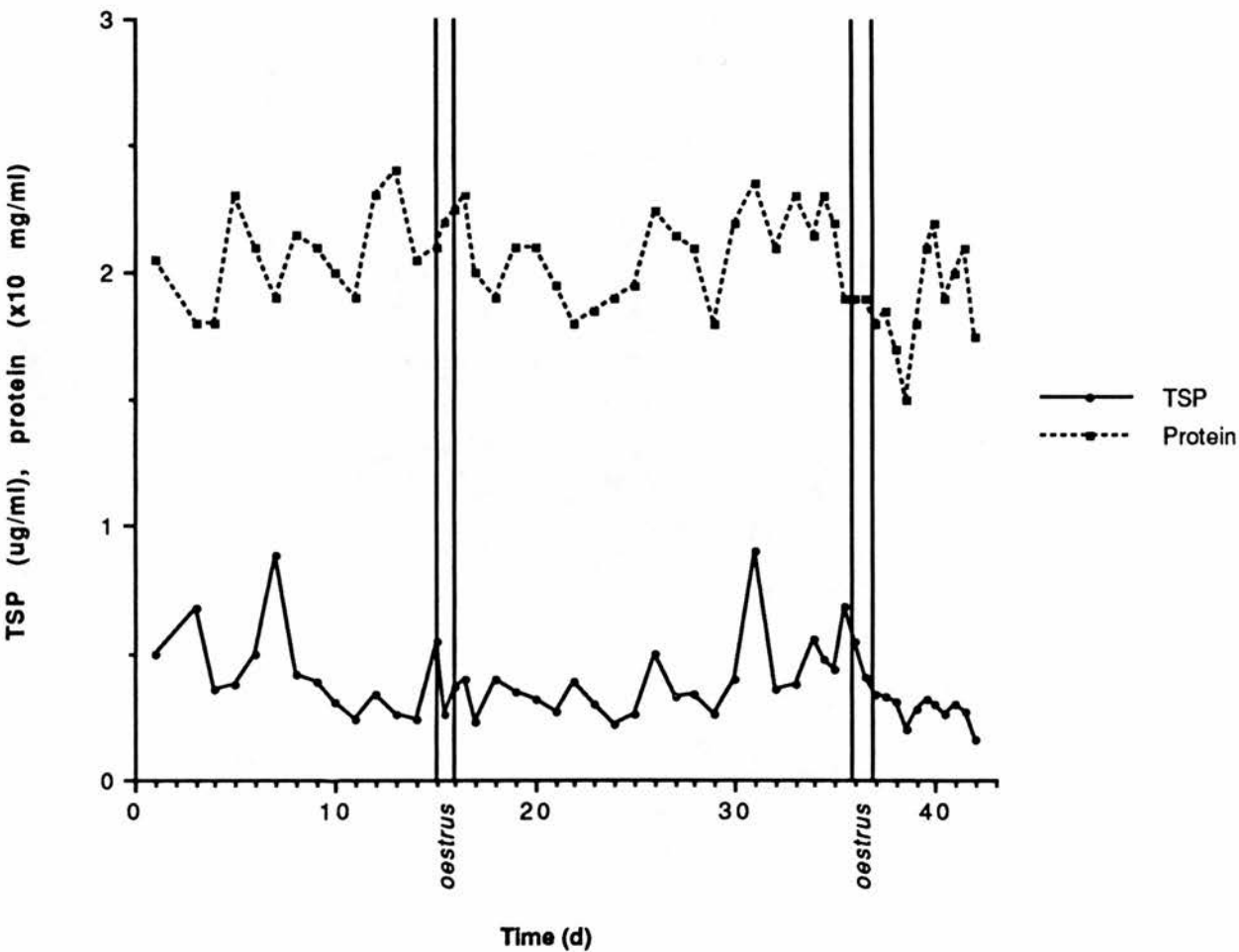


Fig. 5-6. Daily levels of TSP and protein in goat milk before and after mating

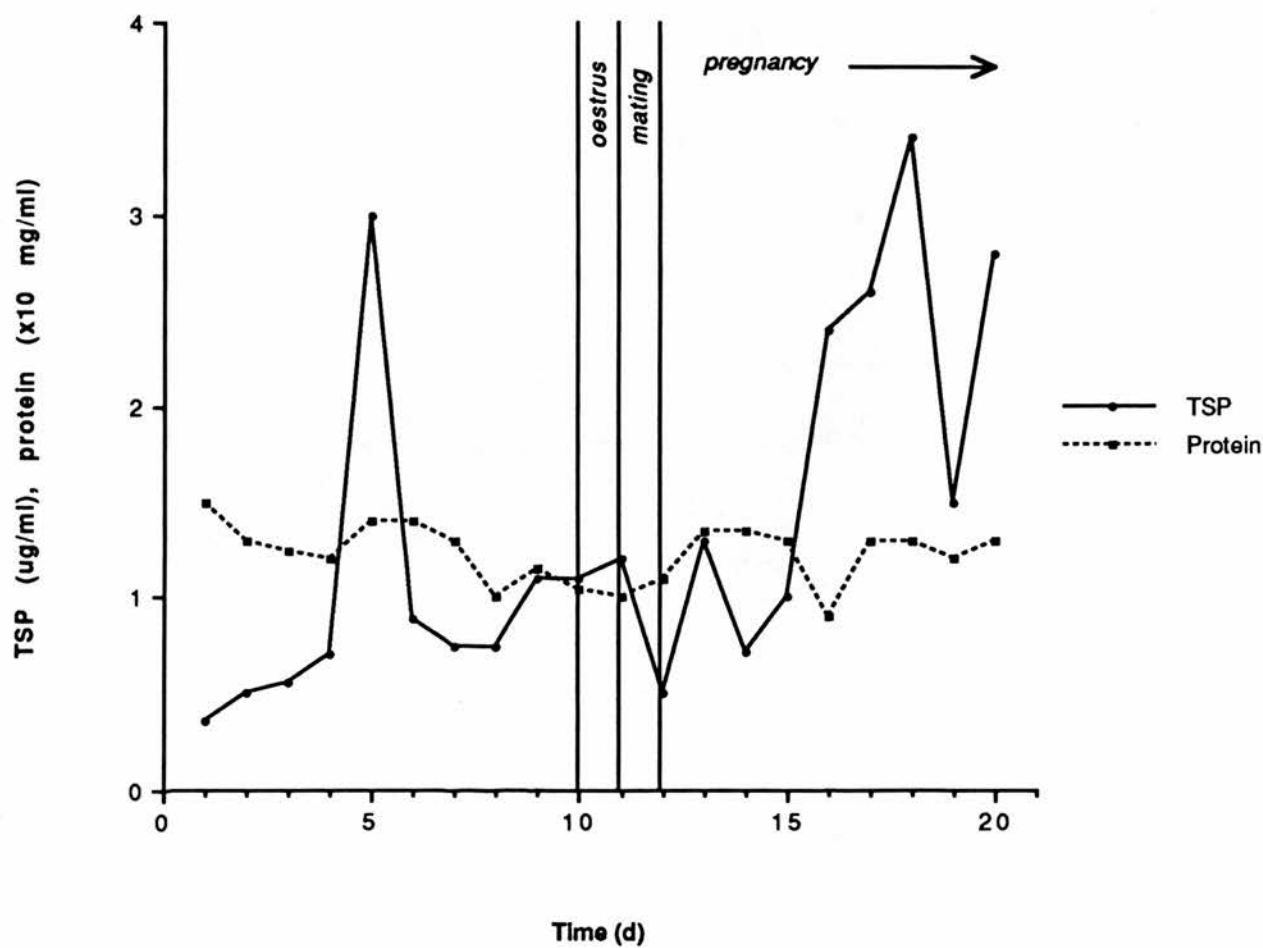
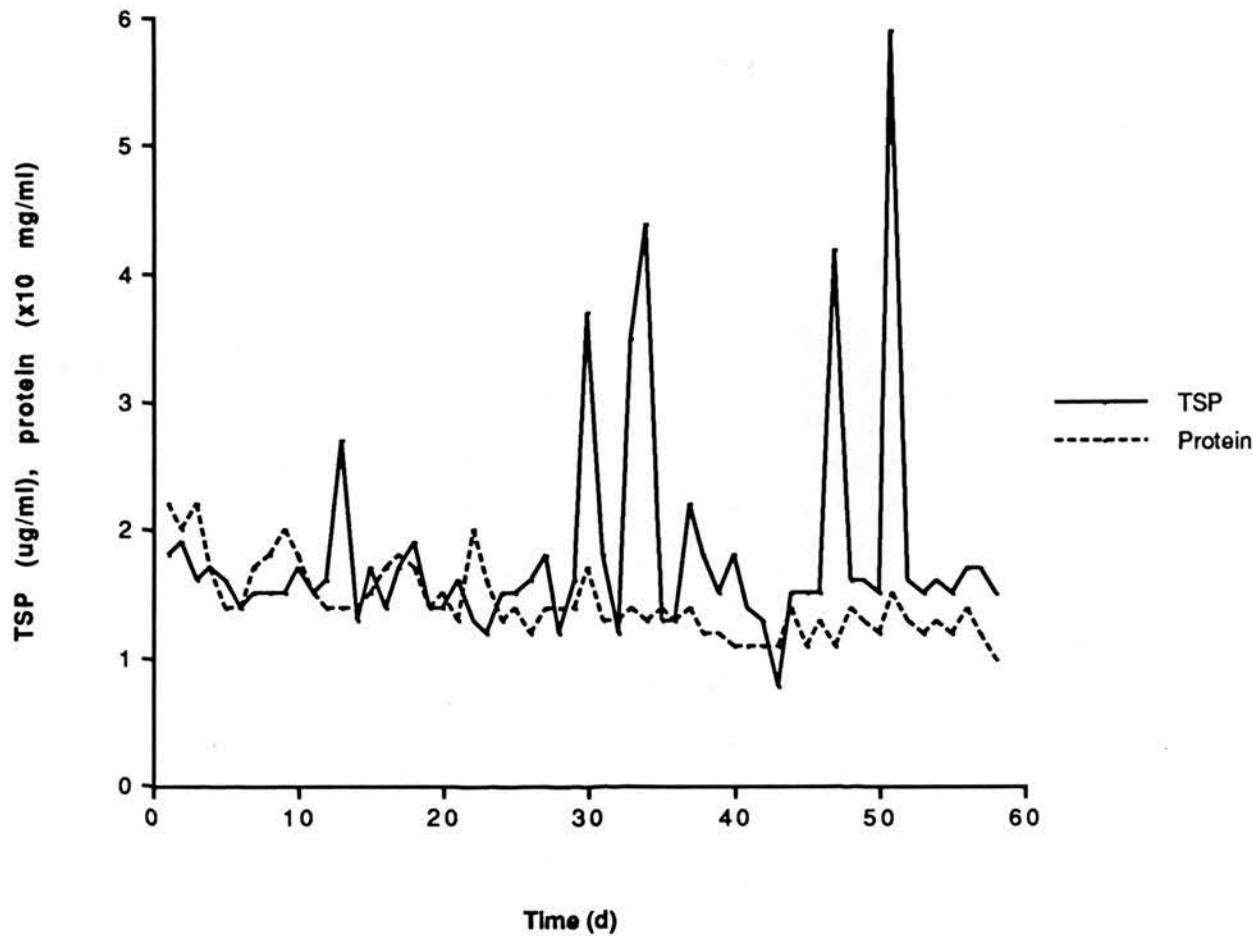


Fig. 5-7. Daily levels of TSP and protein in bovine milk during pregnancy



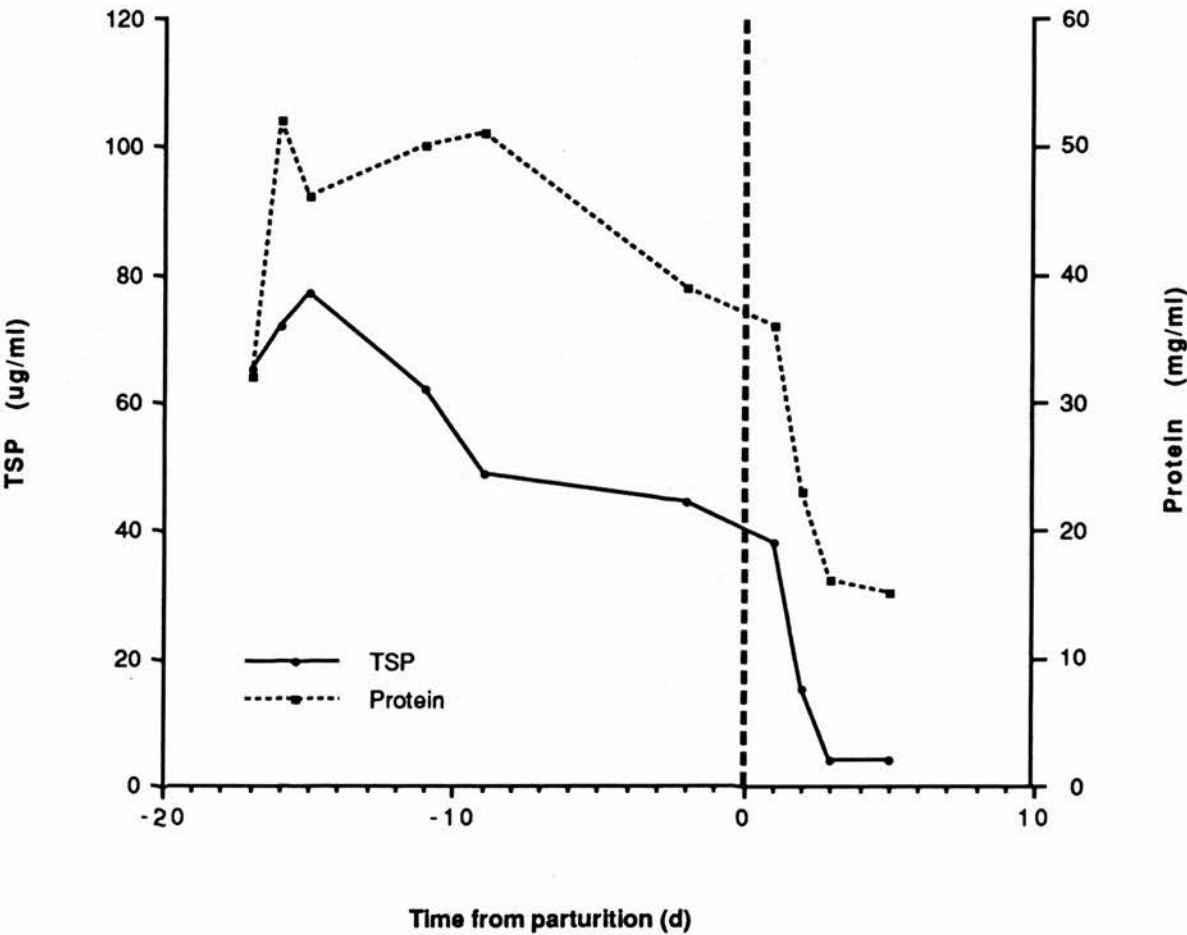
around the time of mating, and from a cow during pregnancy. Goat milk TSP concentrations tended to increase at the onset of pregnancy (fig.5-6), while in the cow a relatively high level of TSP was maintained despite a gradual fall in the total milk protein content as pregnancy progressed (fig.5-7).

#### 5.2.2 Human milk

The concentrations of TSP and protein in mammary secretions of one woman during the period from 17 days prepartum to 5 days postpartum are shown in fig.5-8. Colostrum contained as much as 70 - 80 ug/ml TSP, and this dropped to 4 ug/ml as lactation became established. Total protein levels also fell during the peripartum period but the ratio of TSP to protein declined almost tenfold from 2 to 0.25 ug TSP/mg protein (fig.5-9).

A sample of milk from another lactating woman was assayed for TSP and several other related proteins, and the results are shown in table 5-1. All the proteins were measured in this laboratory by routine radioimmunoassays, except fibrinogen and fibronectin, which were assayed by rocket immunoelectrophoresis. The platelet-specific proteins BTG and PF4 were not detectable in human milk, as was the case for fibrinogen (although the assay for this protein was rather less sensitive). Fibronectin was present at a similar concentration to TSP, while vWF and granulocyte elastase were detectable at low levels.

Fig. 5-8. TSP and protein in human mammary secretions during the peripartum period



g. 5-9. Relative TSP levels in human mammary secretions during the peripartum period

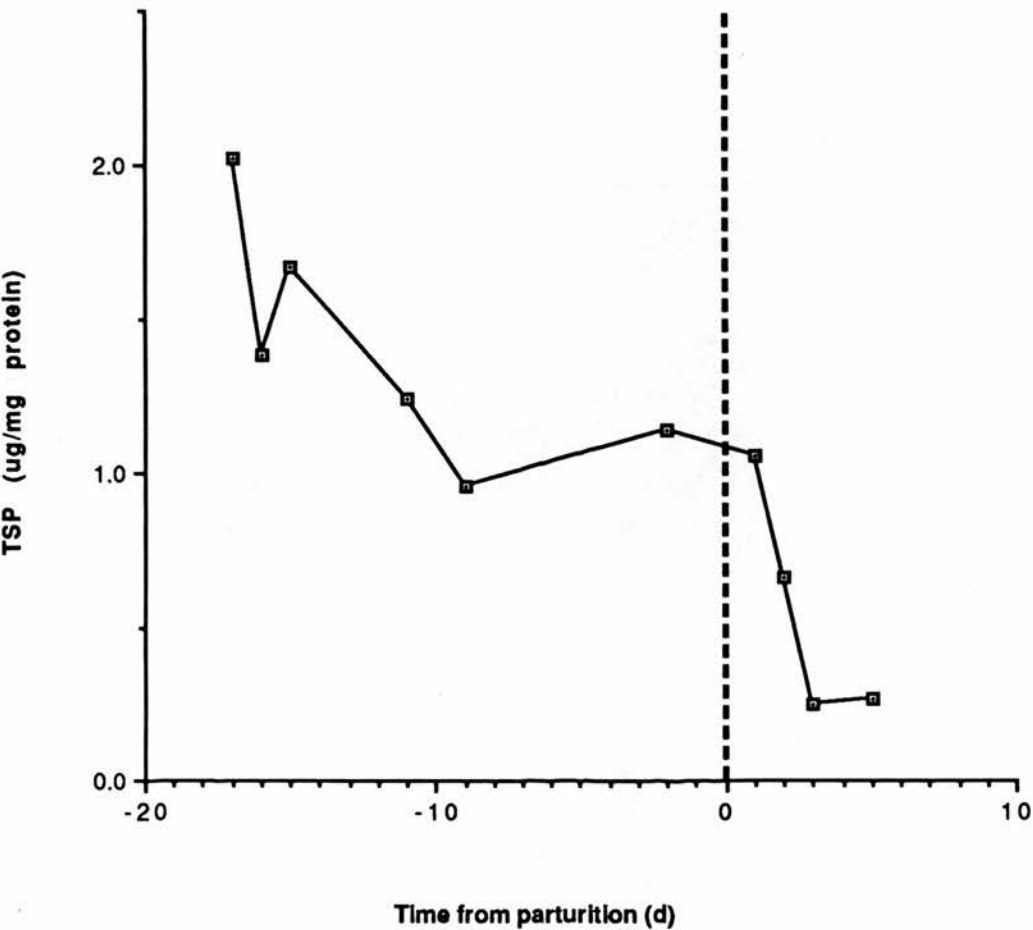


Table 5-1. Levels of platelet-related and adhesive proteins in human milk

Total protein	11.0 mg/ml
TSP	2.3 ug/ml
BTG	< 2.0 ng/ml
PF4	< 5.0 ug/ml
vWF	9.2 ng/ml
Fibrinogen	< 3.0 ug/ml *
Fibronectin	1.6 ug/ml *

Specific proteins measured by radioimmunoassay or \* rocket immunoelectrophoresis



### 5.3 Discussion

In their study of platelet-associated proteins in breast cyst fluids, Miller & Dawes (1985) ruled out platelets as the source of the remarkably high levels of TSP in group II ( $\text{Na}^+$ ) cysts since BTG was not simultaneously elevated above normal plasma concentrations. Group II ( $\text{Na}^+$ ) cysts also contain wholly or predominantly the 7S non-secretory form of IgA, high concentrations of  $\text{Na}^+$ , IgG and albumin and low levels of  $\text{K}^+$  and DHA-sulphate (Miller et al 1983a, Yap et al 1984); findings which are consistent with the hypothesis that these fluids may be formed by accumulation from plasma. In contrast, group I ( $\text{K}^+$ ) cysts contain the 11S secretory form of IgA, low concentrations of  $\text{Na}^+$ , IgG and albumin and high levels of  $\text{K}^+$  and DHA-sulphate; suggesting that these fluids may reflect the synthetic activity of the apocrine epithelium. These concepts of fluid generation are supported by comparison of the epithelial lining of cysts; group I ( $\text{K}^+$ ) cysts are characterised by active apocrine epithelium, whereas group II ( $\text{Na}^+$ ) cysts possess thin, attenuated (flattened) epithelial cells (Dixon et al 1983). Since TSP concentrations are highest in group II ( $\text{Na}^+$ ) cysts, it seems very unlikely that these levels represent synthesis by apocrine cells. Alternatively, TSP may be actively accumulated or produced locally by other cells associated with breast cysts.

The present data confirm the finding by Miller & Dawes (1985) of high levels of TSP in human breast cyst fluids, particularly those of group II. Furthermore, an inverse relationship has been shown to exist between TSP and EGF concentrations in cyst fluids. This negative correlation is probably due to differences in fluid composition between the two groups of cysts, since EGF levels were

lower in group II fluids, as has recently been reported (Smith et al 1989). EGF has been detected in cyst fluid by several others (Jaspar & Franchimont 1985, Collette et al 1986, Boccardo et al 1988) and also in milk (Starkey & Orth 1977) and in nipple aspirates (Rose 1986).

The higher concentrations of EGF in group I fluids may reflect the active nature of the apocrine epithelium surrounding these cysts. Alternatively, the differences in EGF content may be responsible for the varying epithelial activities of group I and group II cyst walls. EGF has been shown to have potent mitogenic effects on breast epithelial cells (Osborne et al 1980, Imai et al 1982), while Dickson and coworkers (1985) have reported the secretion of EGF-like peptides by breast cancer cell lines. Although there is no direct evidence that breast cyst epithelium produces EGF, mammary epithelium can secrete EGF in vivo which has been administered systemically (Brown et al 1986). Taken together, the above data could imply that EGF in breast cyst fluids is produced by the lining epithelium and may potentially modulate the activity of these cells by autocrine/paracrine mechanisms. TSP levels, on the other hand, were found to correlate inversely with EGF in breast cysts, and it may be that mammary epithelial cells are not the major source of TSP in these secretions.

Separation of a group II ( $\text{Na}^+$ ) cyst fluid by heparin affinity chromatography showed that although some of the TSP detected by radioimmunoassay retained a heparin-binding affinity similar to that of platelet TSP (eluting with 0.55 M NaCl), most was eluted in the initial column flow-through together with the measurable cyst fluid protein content, plus another peak of reduced heparin-binding

affinity (eluting with 0.25 M NaCl). The loss of heparin-binding affinity may be due to partial degradation of TSP by proteases, or to the association of TSP with other molecules in cyst fluid.

TSP was found in high concentrations in the aqueous phase of milk (constituting 0.2% and 0.02% of the total protein of colostrum and mature milk respectively) compared with plasma (where it accounts for only 0.00015% of the total protein). Data from animal models suggested that the relative TSP content of milk tended to increase with time during pregnancy, when the mammary gland becomes fully developed, i.e. hypertrophy of the glandular epithelium and differentiation of acini into milk-secreting units occurs.

However, the pattern of TSP secretion by the human breast observed during the initiation of lactation was the inverse of that described for the specific milk proteins  $\alpha$ -lactalbumin and lactoferrin (Kulski & Hartmann 1981). Thus, compared to the total protein content, the relative TSP concentration decreased tenfold during the transition from colostrum to mature milk, whereas the relative levels of  $\alpha$ -lactalbumin and lactoferrin increase over this period.  $\alpha$ -Lactalbumin is synthesised by differentiated mammary epithelial cells (Hill & Brew 1975) and while lactoferrin is probably synthesised in situ by the breast, additional amounts may be produced by white blood cells which invade its interstitial tissues (Rumke et al 1971).

It is of considerable interest that the pattern of TSP secretion in early milk closely resembled that of IgA, the major immunoglobulin of milk. IgA also shows a dramatic fall in concentration during the first few days after parturition (Ammann & Stiehm 1966). Human milk contains around  $1 - 4 \times 10^6$  white blood

cells/ml during the first two weeks following delivery and slightly fewer thereafter (Hanson & Winberg 1972). These white cells include lymphocytes (which secrete IgA), macrophages and neutrophils, and have also been described in nipple aspirate fluid (Petrakis 1986). In the present study, granulocyte elastase (a product of neutrophils) was found in human milk along with TSP, but the platelet-specific proteins BTG and PF4 could not be detected. It is apparent that the composition of milk and other breast secretions may be influenced by cells other than those of the mammary epithelium. Since TSP is known to be secreted by macrophages (Jaffe et al 1975), there is a strong possibility that the high levels of TSP in breast secretions may originate from these and/or other cells which infiltrate the mammary gland, and not from platelets or the glandular epithelium itself.

## CHAPTER SIX

### THROMBOSPONDIN IN MALIGNANT AND NON-MALIGNANT BREAST TISSUE

TSP was found in high concentrations in secretions of the breast (chapter 5) and therefore it was interesting to study its levels in breast tissue, with the aim of investigating further the origin and production of TSP in the mammary gland. Measurements of TSP were made in two sets of breast tissue cytosols. Firstly, TSP concentrations were compared within benign and malignant breast tissue, with the corresponding contents of BTG and vWF, and with various indicators of hormonal status. Secondly, levels of TSP were related to the site of the tissue with respect to the tumour mass, and to the concentration of tissue plasminogen activator (tPA), an oestradiol-inducible enzyme. The results of these studies have been published (Pratt et al 1989).

Breast tissue cytosols (101 breast carcinoma, nine benign mammary dysplasia, five fibroadenoma, one gynaecomastia, one normal) were provided by Miss J. Telford, University Department of Clinical Surgery, Edinburgh, and had been prepared as described in section 2.7. Nineteen breast tissue cytosols from eight patients with breast cancer, which were classified according to whether the tissue had been taken from the centre of the tumour ( $n = 7$ ), the edge of the tumour ( $n = 3$ ), or the surrounding non-malignant tissue ( $n = 9$ ), were provided by Dr G. Savidge, Department of Haematology, St. Thomas' Hospital, London. They had been obtained by

snap-freezing of washed tissue samples taken at mastectomy, followed by cryofragmentation using a mechanical dismembrator, and preparation of a supernatant from the tissue extract resuspended in buffered saline.

#### 6.1 TSP, vWF and BTG in breast cytosols

A wide range of TSP levels was found in breast cytosols (fig.6-1). Highest levels were observed in breast cancers ( $n = 101$ , median = 317 ug TSP/g cytosol protein, range = 17.4 - 23400 ug TSP/g cytosol protein) and lowest in benign breast ( $n = 15$ , median = 21.7, range = 1.78-155). The difference between cancer and benign samples was highly discernible ( $P < 10^{-6}$ , Wilcoxon-Mann-Whitney test). TSP in fibroadenomas ( $n = 5$ , median = 21.7, range = 5.93 - 155) was not statistically discernible from that in benign mammary displasia and gynaecomastia ( $n = 10$ , median = 19.7, range = 1.78 - 47.6) and therefore all benigns were considered as a single group.

Measurement of vWF in breast cytosols revealed a narrower range of concentrations compared with TSP (fig.6-2), but higher levels were also found in cancers ( $n = 101$ , median = 40.8 ug vWF/g cytosol protein, range = undetectable - 392 ug vWF/g cytosol protein) than in benigns ( $n = 15$ , median = 14.0, range = undetectable - 46.0). The difference was statistically discernible ( $P < 10^{-4}$ ) although less marked than for TSP. A plot of TSP against vWF (fig.6-3) showed that there was a positive correlation between the two which was statistically discernible ( $n = 116$ ,  $r_s = 0.484$ ;  $P < 0.001$ , Spearman rank correlation test).

BTG was measured together with TSP in 20 breast cytosols (18 cancer, one benign, one normal) and no statistically discernible

Fig. 6-1. TSP in breast cancer and benign breast tissue

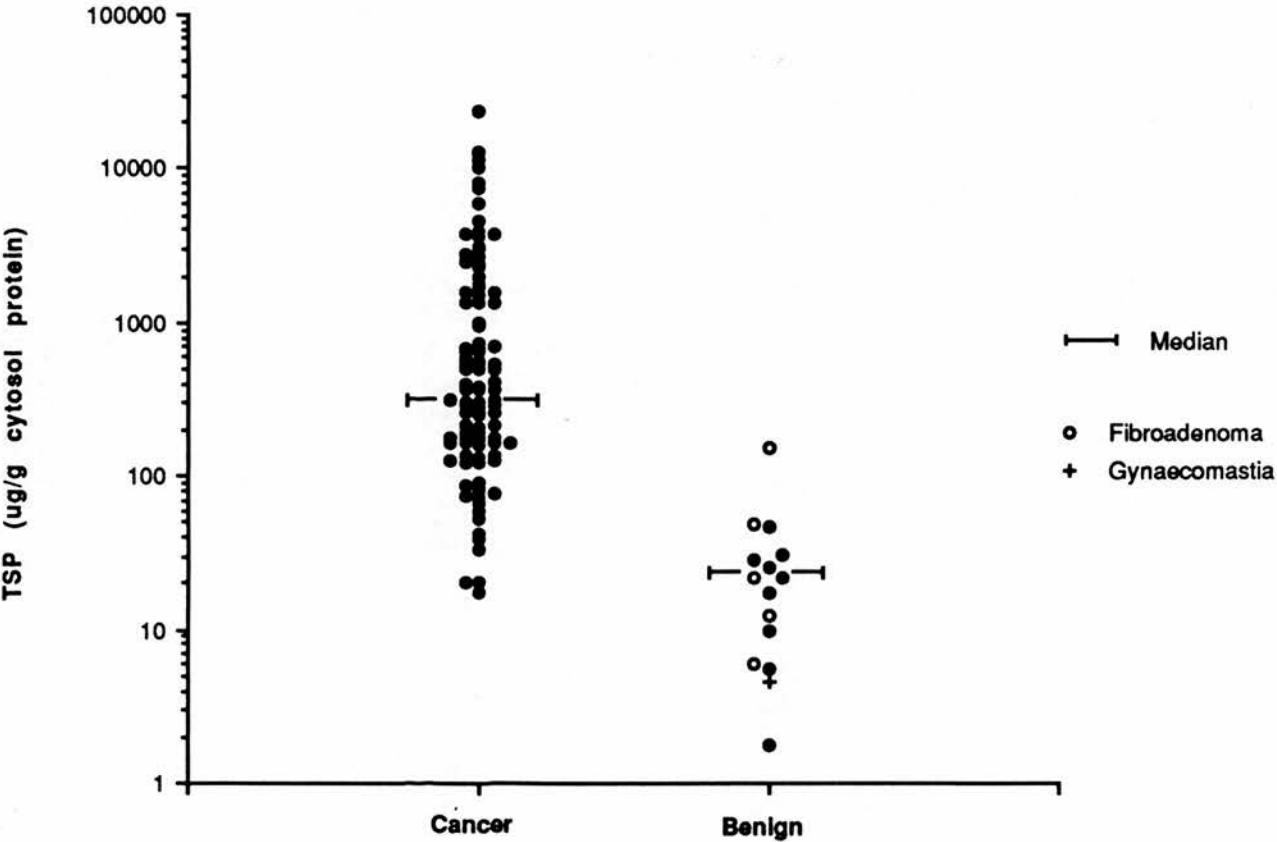


Fig. 6-2. vWF In breast cancer and benign breast tissue

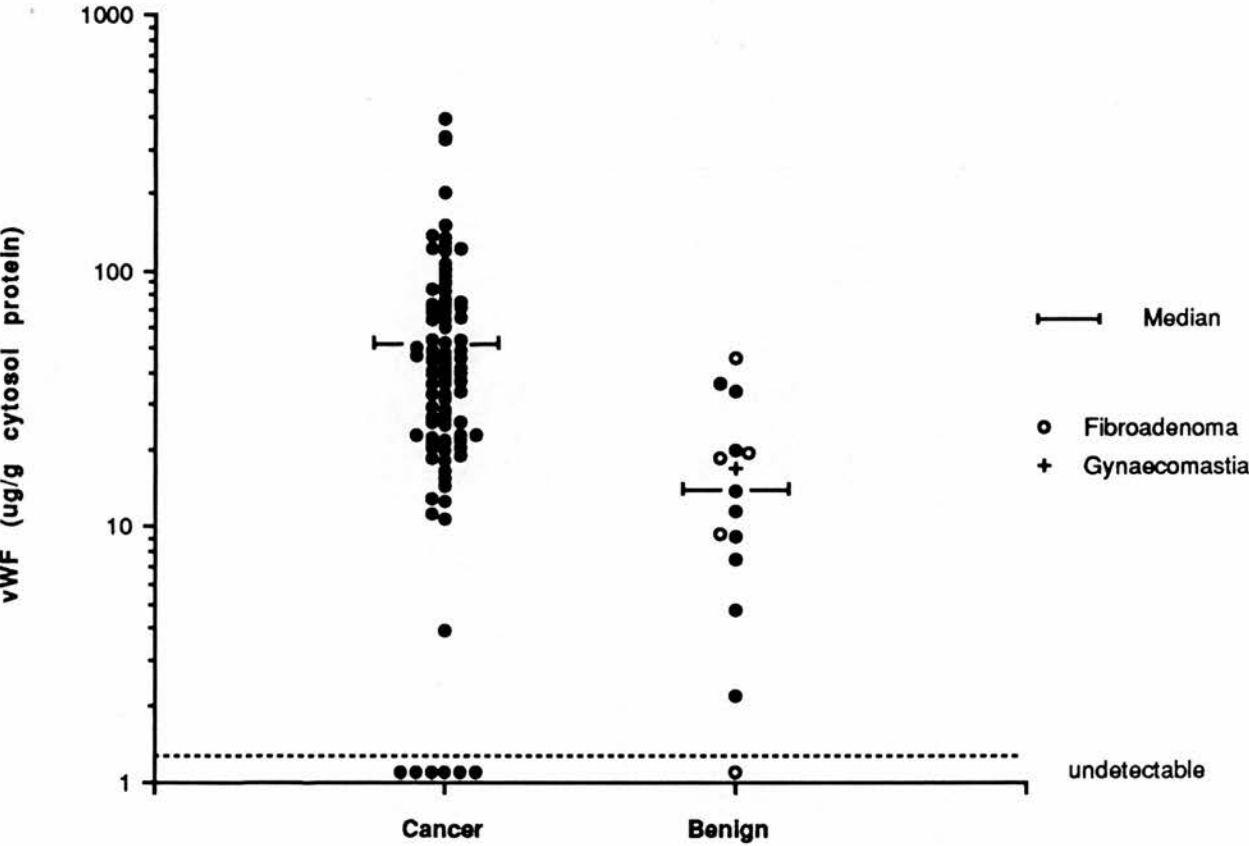




Fig. 6-3. Relationship between TSP and vWF in breast tissue

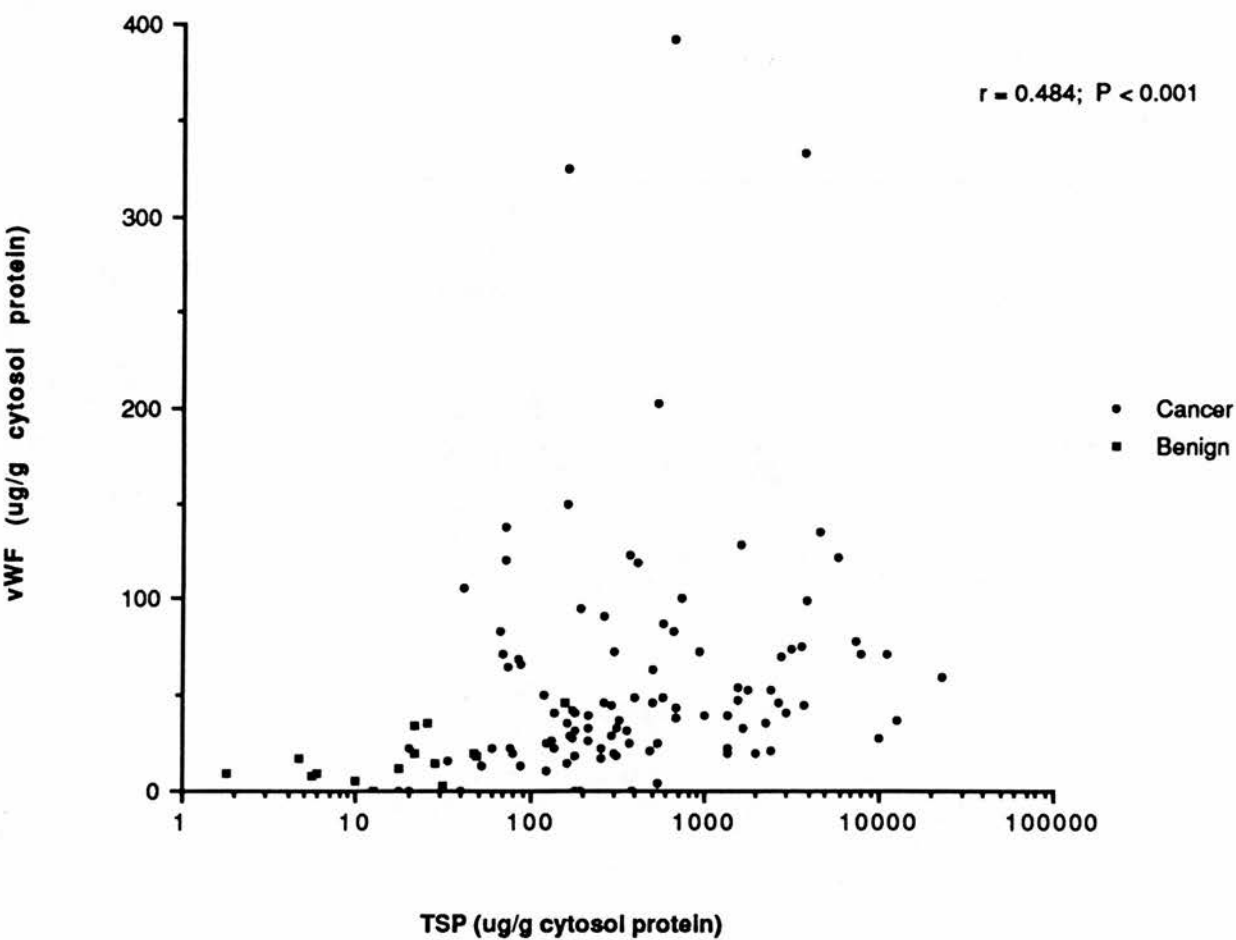
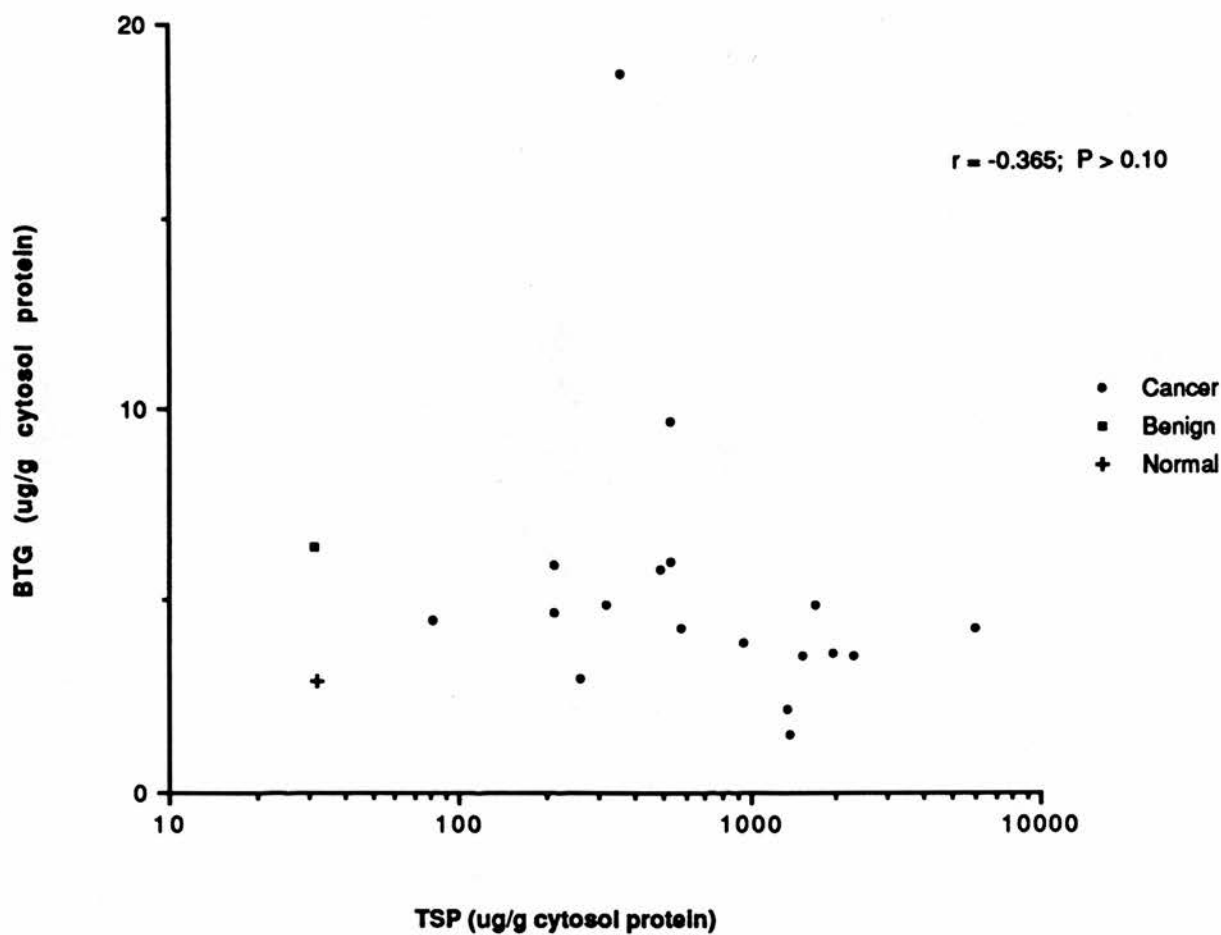


Fig. 6-4. Relationship between TSP and BTG in breast tissue



correlation was observed between the two ( $r_s = -0.365$ ;  $P > 0.10$ ) (fig.6-4).

## **6.2 TSP and indicators of hormonal status in breast cancer cytosols**

Breast cancer cytosol TSP levels were compared in a group of 92 patients classified according to menopausal status (fig.6-5). TSP in breast cancer cytosols from pre-menopausal women ( $n = 32$ , median = 213.5 ug TSP/g cytosol protein, range = 17.4 - 23400 ug TSP/g cytosol protein) was slightly lower than that in cancers from post-menopausal women ( $n = 60$ , median = 367, range = 41.8 - 12800), the difference was just statistically discernible ( $P = 0.043$ ).

TSP did not correlate with oestrogen receptor (ER) concentration in breast cancers (fig.6-6), measured in the University Department of Clinical Surgery, Edinburgh using a method described by Hawkins et al 1981 ( $n = 98$ ,  $r_s = -0.0075$ ;  $P > 0.20$ ) nor with progesterone receptor (PR) concentration (fig.6-7), measured by the method of Miller et al 1983b ( $n = 33$ ,  $r_s = -0.146$ ;  $P > 0.20$ ).

## **6.3 TSP and tissue plasminogen activator (tPA) in cytosols of tissue taken from different locations within cancerous breast**

Nineteen breast tissue cytosols from eight patients with breast cancer were measured for their TSP and tPA content (results from the tPA enzyme-linked immunosorbant assay, as described by Rijken et al 1984, have been published by Layer et al 1987). The tissue had been classified according to whether it was taken from the centre of the tumour mass ('centre'), from the edge of the tumour ('interface') or from the surrounding non-malignant tissue

Fig. 6-5. Relationship between menopausal status and TSP in breast cancer tissue

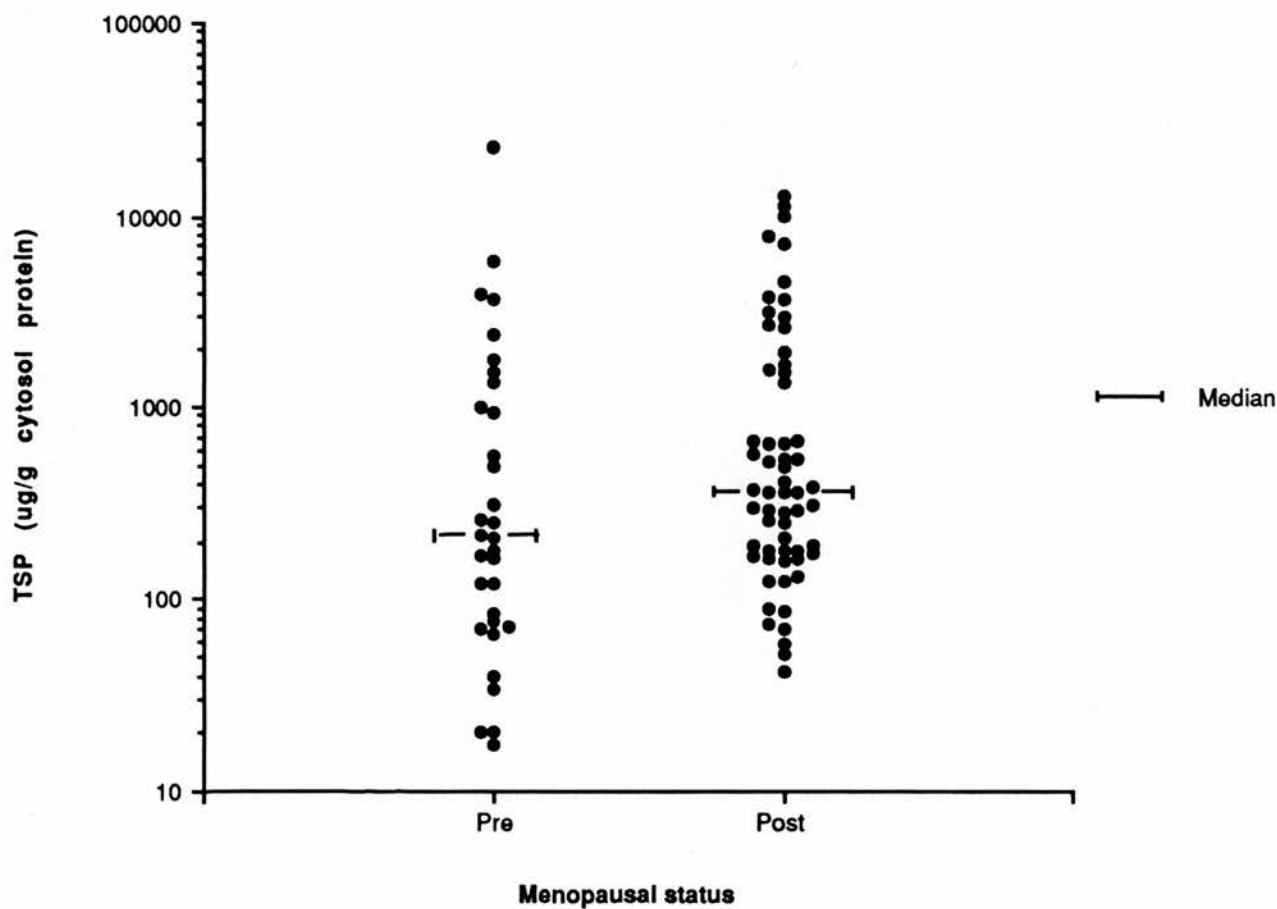


Fig. 6-6. Relationship between TSP and ER in breast cancer tissue

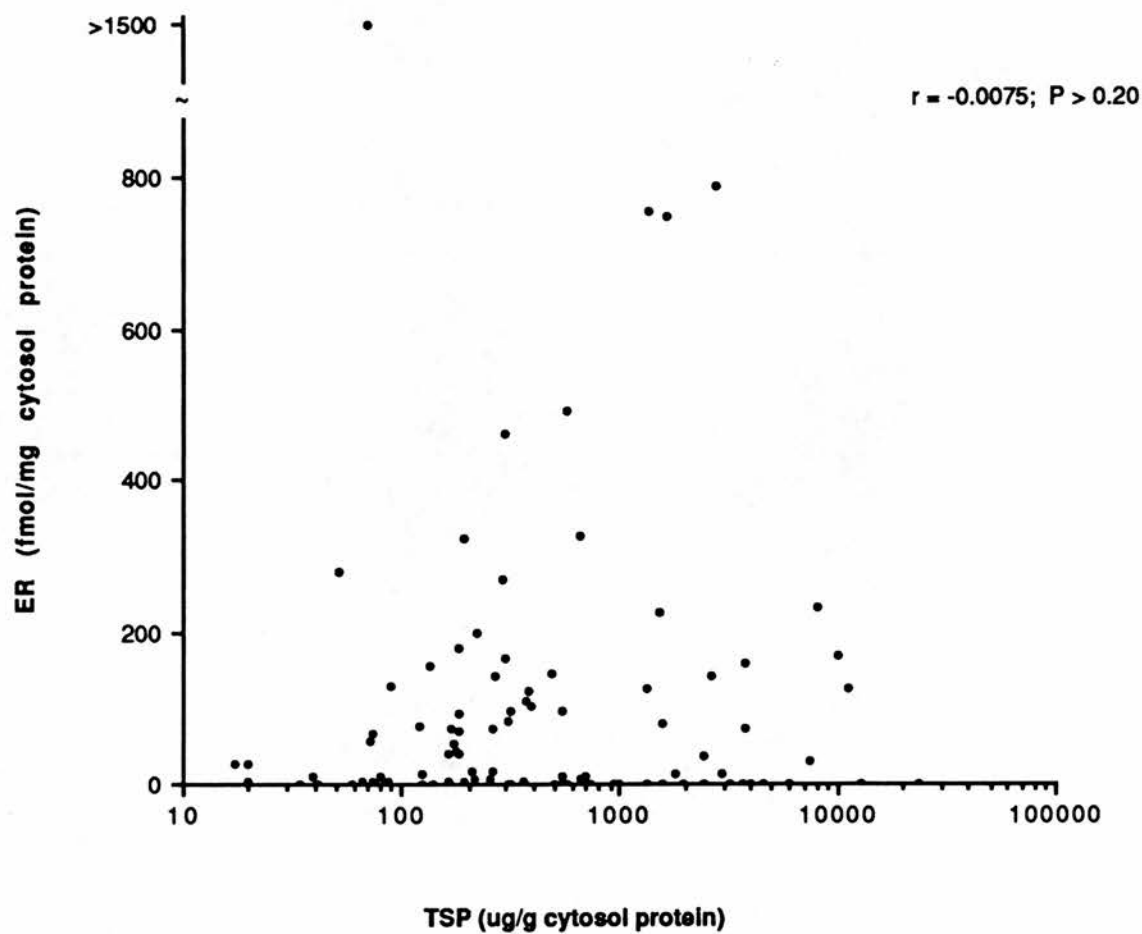
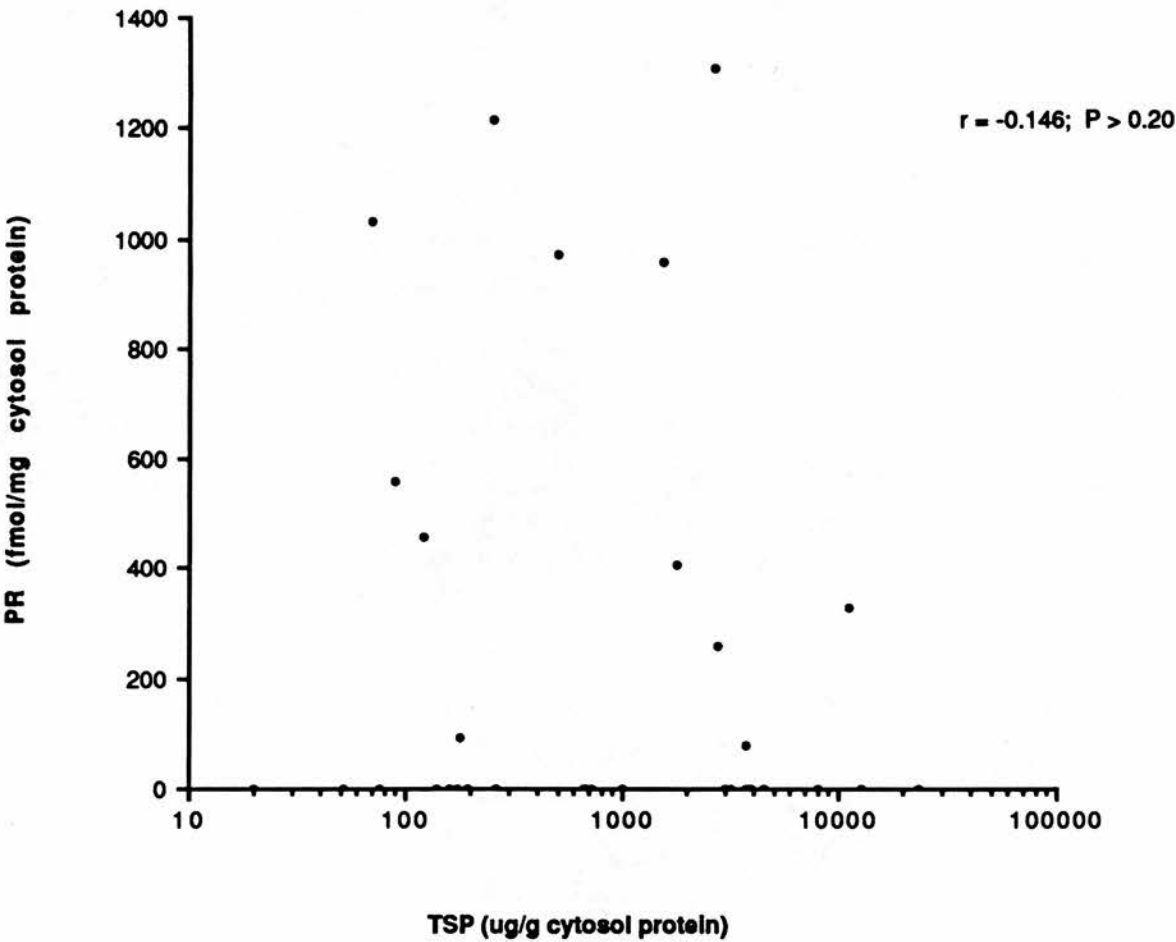


Fig. 6-7. Relationship between TSP and PR in breast cancer tissue



('surround').

A weak negative correlation which was not statistically discernible was observed between TSP and tPA in breast tissue ( $n = 19$ ,  $r_s = -0.356$ ;  $P > 0.10$ ) (fig.6-8).

However, comparison of the TSP concentration in samples from the centre of the tumour mass and from the surrounding non-malignant tissue (fig.6-9) showed that the centre ( $n = 7$ , median = 1080 ug TSP/g cytosol protein, range = 31.8 - 4480 ug TSP/g cytosol protein) contained much higher levels than the surround ( $n = 9$ , median = 53.0, range = 17.0 - 324). The difference between the two groups was statistically discernible ( $P < 0.01$ ), and in three out of four patients where samples from both locations were available, TSP was higher in the malignant tissue.

Conversely, tPA concentrations were lower in the centre ( $n = 7$ , median = 3.19 ug tPA/g cytosol protein, range = 1.30 - 4.39 ug tPA/g cytosol protein) than in the surround ( $n = 9$ , median = 5.42, range = 2.50 - 11.99) (fig.6-10). The difference between the two groups was statistically discernible ( $P < 0.005$ ), and in all four patients where samples from both locations were available, tPA was lower in the malignant tissue.

#### 6.4 Discussion

The elevated levels of TSP and vWF in malignant breast, and the correlation observed between the two suggest that they may have a common site of synthesis within the breast which is stimulated in malignant disease. Whilst vWF is not known to be associated with cancer, TSP has been implicated previously in tumour cell metastasis (section 1.8).

Fig. 6-8. Relationship between TSP and tPA in breast tissue

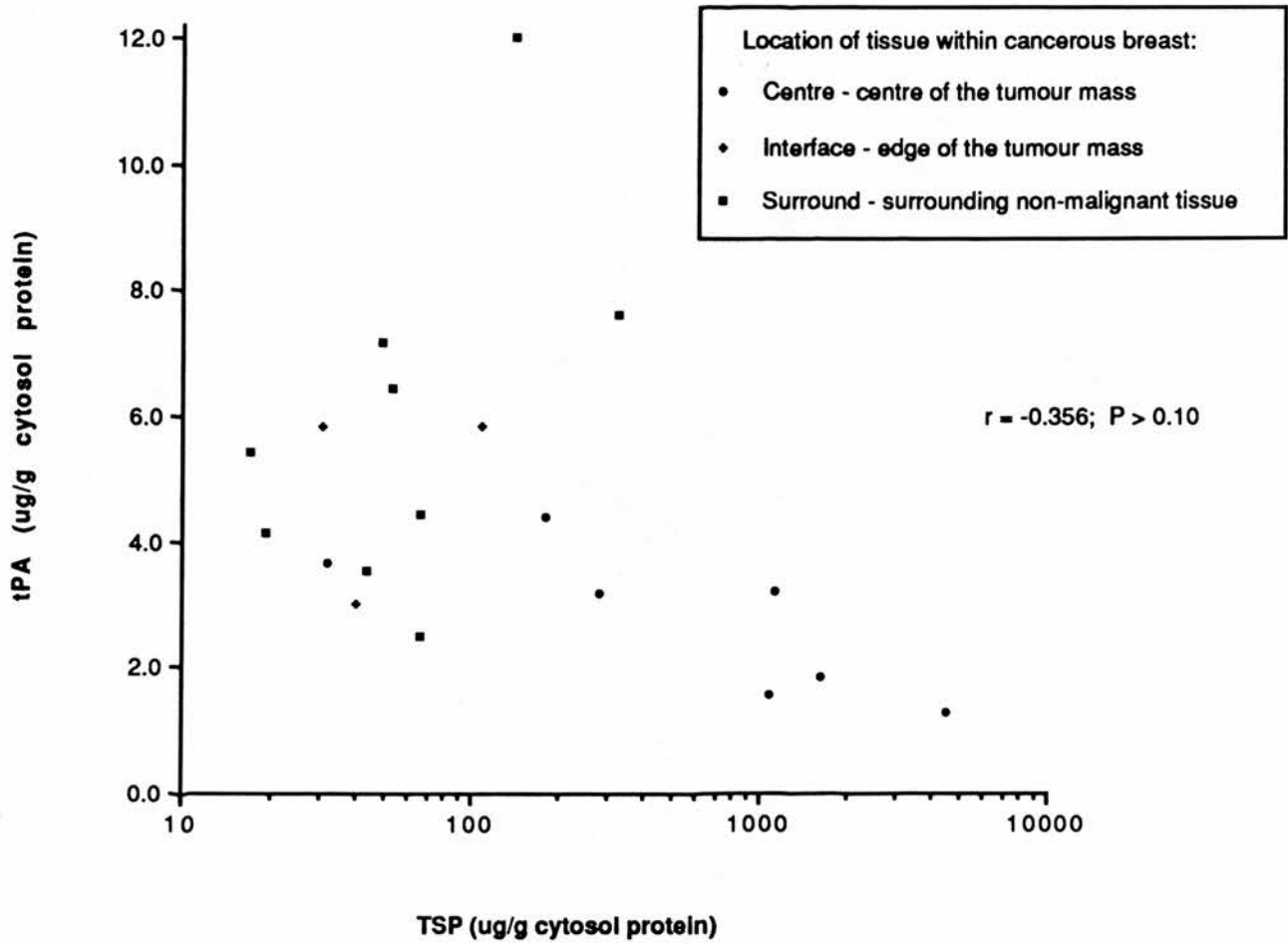




Fig. 6-9. TSP in breast tissue from different locations within cancerous breast

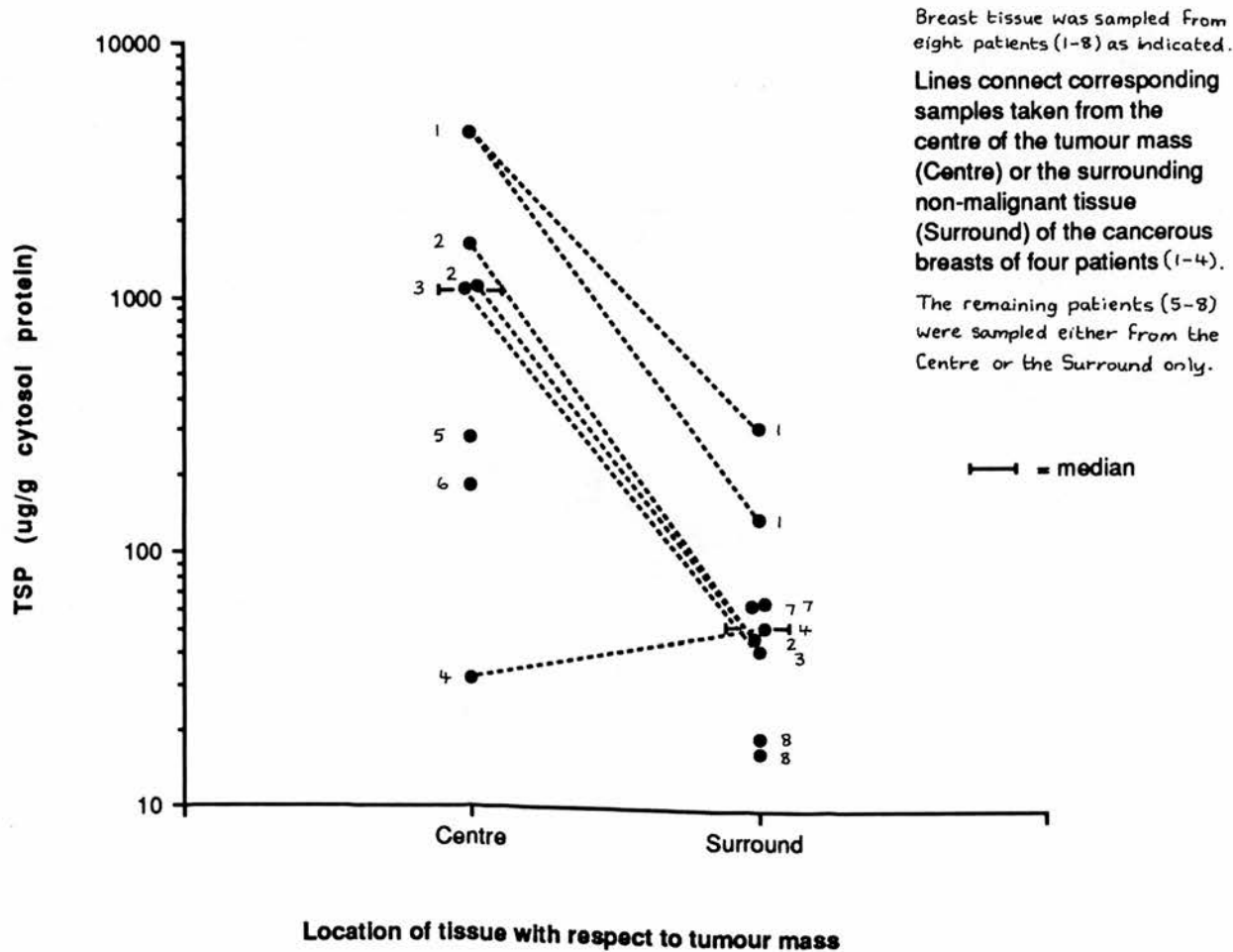
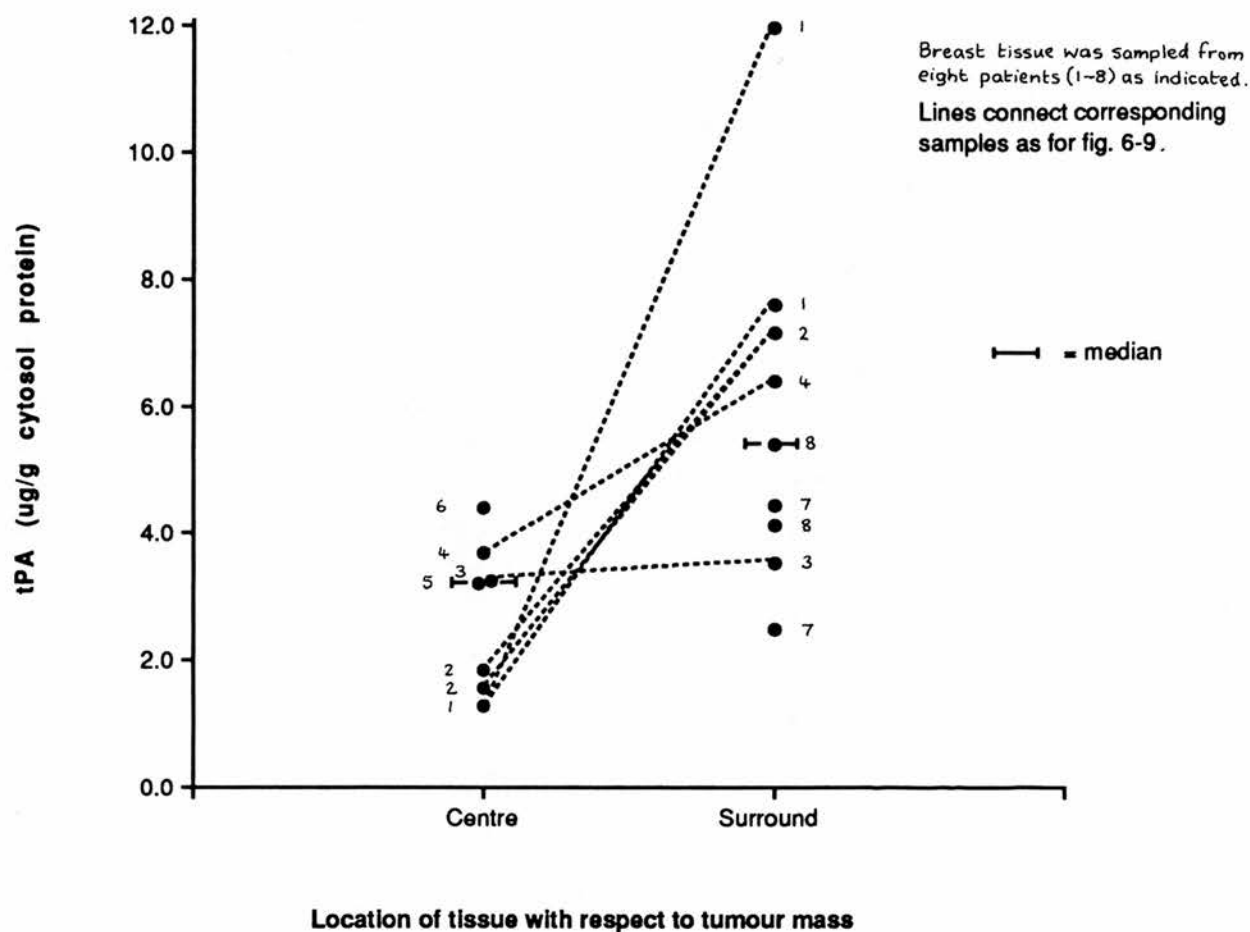


Fig. 6-10. tPA in breast tissue from different locations within cancerous breast



vWF is synthesised by endothelial cells and megakaryocytes and is contained in platelet  $\alpha$ -granules. vWF functions as a carrier protein for coagulation factor VIII in plasma and is thought to be involved in platelet-vessel wall interactions (Girma et al 1987). Indeed, vWF is incorporated into the extracellular matrix of cultured endothelial cells (Hormia et al 1983) where it may function as an adhesive protein. Although not specific for endothelial cells, vWF has been used as a marker of endothelial cell perturbation in vitro (Mannucci et al 1975, Levine et al 1982, Loesberg et al 1983) and as a criterion for endothelial cell identification (Jaffe et al 1973).

TSP is also synthesised by endothelial cells and megakaryocytes, and is stored in platelet  $\alpha$ -granules, but has also been described in a wide range of other cell types (section 1.5). Since the platelet  $\alpha$ -granule protein BTG was not simultaneously raised with TSP in breast cytosols, it is very unlikely that platelets are the source of the high levels of TSP and vWF in malignant breast. Increased vascularity and/or stimulation of the endothelium could explain the higher TSP and vWF content of cancer tissue despite the finding that the difference between cancer and benign samples was much greater for TSP than for vWF (the median cytosolic vWF of malignant breast was three times higher than non-malignant breast, whereas for TSP it was 15 times higher). TSP and vWF have been shown to be located in different subcellular compartments in endothelial cells where their secretion is separately controlled (Reinders et al 1985). However, it is possible that another source within the breast, in addition to the endothelium, may be contributing to the very high level of TSP observed in some breast

cancers.

It has been said that breast cancer is hormonally mediated and that oestrogens are the prime agents in tumour expression (Miller & Bulbrook 1980). Approximately 30% of breast cancers respond to oestrogen deprivation therapy and most of these are ER positive (Edwards et al 1979). Since oestrogens are so closely implicated in breast cancer, it was of interest to investigate whether the high levels of TSP in malignant breast were related to any of the known indicators of hormonal status. A slight difference was observed in the TSP content of breast cancers from pre- and post-menopausal women, but no correlation was observed between TSP and ER or PR, and therefore it seems unlikely that synthesis of TSP by the malignant epithelium regulated by oestrogens is involved in causing the high levels found in breast cancer.

Further evidence for the specific elevation of TSP in malignant breast came from the measurement of TSP in tissue taken from different sites within the cancerous breasts of a group of patients. Very high levels of TSP ( $>500$  ug/g cytosol protein) were only seen in tissue from the centre of the tumour mass; levels in tissue from elsewhere within the same breast were comparable to those found in benign breast.

It was interesting that measurement of tPA in these samples showed the opposite trend. tPA is the major plasminogen activator (PA) in human milk where it shows a similar pattern of secretion during early lactation as does TSP (Marshall et al 1986, section 5.2.2). While elevated PA levels have been reported in breast cancer (Dano et al 1985), some tumours contain predominantly urokinase-type plasminogen activator (uPA) and others predominantly

tPA (O'Grady et al 1985) which makes early studies in which only total PA was measured difficult to interpret. However, it has been established that tPA, rather than uPA, correlates with ER and PR in breast tumour cytosols (Duffy et al 1986) and tPA secretion by mammary epithelial cells in culture is preferentially induced by oestradiol and glucocorticoids (Ryan et al 1984, Busso et al 1986). The finding that TSP and tPA were inversely related in tissue from different sites within cancerous breasts suggests that they are not coordinately expressed by the malignant epithelium.

## CHAPTER SEVEN

### THROMBOSPONDIN IN CULTURED CELLS

In vitro culture of cells grown on plastic surfaces in media of defined composition was used as a model system in which to study the production of TSP by cells derived from various human tissues. Three main types of human cells were investigated; endothelial cells, fibroblasts and an established breast cancer cell line (MCF-7) of epithelial origin. Secretion of TSP into the extracellular medium was quantitated with respect to time for each cell type, and comparisons were made between endothelial cells and fibroblasts from various tissue sources. Factors which may potentially influence the production of TSP by endothelial and MCF-7 cells were also studied.

In the following experiments, unless stated otherwise, cells were grown in 6-well Costar culture clusters (Northumbria Biologicals) using complete medium (2 ml per well) as described in section 2.11. At confluence, the monolayer was washed with buffered salt solution to remove residual serum components, and incubated with serum-free medium (SFM) for the experimental time period. Supernatants, prepared by aspiration of the serum-free conditioned medium (SFCM) and centrifugation to remove cell debris, were stored at  $-40^{\circ}\text{C}$  prior to assay. Cell numbers were measured using a haemocytometer following trypsinisation of the monolayer. Some of the experiments were performed in collaboration with

Dr N.R. Hunter, Scottish National Blood Transfusion Service  
Headquarters Unit Laboratory, Edinburgh.

## **7.1 Endothelial cells**

### **7.1.1 Secretion of TSP by endothelial cells derived from human umbilical vein (HUVEC) and adult saphenous vein (HSVEC)**

The secretion of TSP by HUVEC and HSVEC has been measured in this laboratory and the results described by Hunter et al (1984). HUVEC and HSVEC both secreted TSP into the culture medium in a linear time-dependent manner with no apparent feedback control. After 24 and 48 h, HUVEC were found to produce about 5 times as much TSP as HSVEC (approximately 6 ug TSP/ $10^6$  cells compared with 1.2 ug TSP/ $10^6$  cells at 24 h).

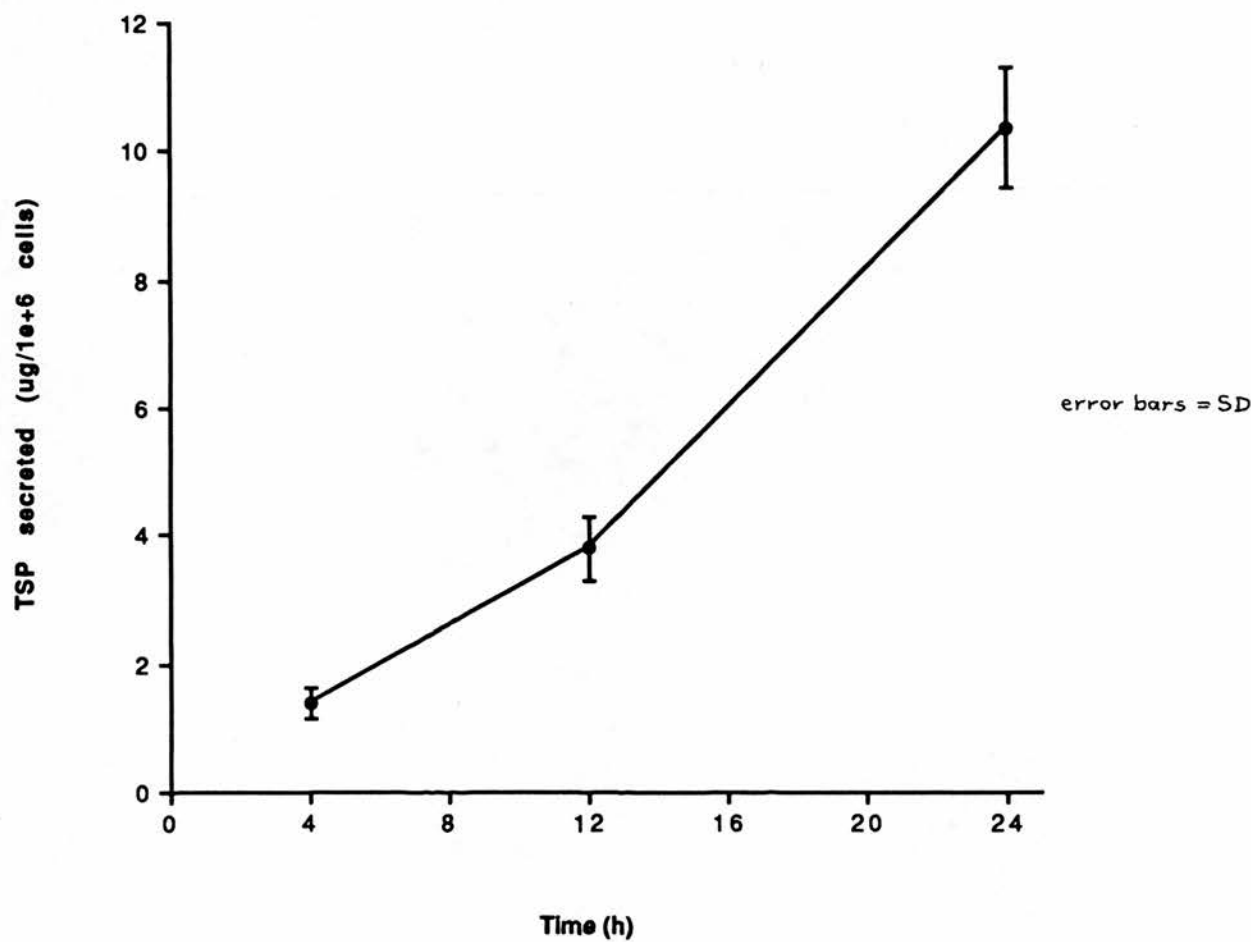
### **7.1.2 Secretion of TSP by human umbilical artery endothelial cells (HUAEC)**

An endothelial cell line was isolated from human umbilical cord artery as described in section 2.11.1.1. SFCM was removed at 4, 12 and 24 h (8 wells for each time point) for measurement of TSP, as shown in fig.7-1. HUAEC were found to secrete almost twice the amount of TSP as their venous counterparts (10.4 ug TSP/ $10^6$  cells/24 h).

### **7.1.3 Effect of cell density on TSP secretion by HUVEC**

A HUVEC line was plated at 25, 50, 75 and 100% confluent density (6 wells for each density) and incubated for 2 h in complete medium. The monolayer was washed, the cells incubated in SFM for

**Fig. 7-1. TSP secretion by human umbilical artery endothelial cells (HUAEC)**





12 h, and the supernatants assayed for TSP as before. Cell numbers were measured in 4 wells at each density, except for the 25% confluence group for which an accurate cell count could not be determined because of the low cell number. Fig.7-2 illustrates the secretion of TSP measured in the same HUVEC line plated at different cell densities. Results have been expressed (i) as the amount secreted per  $10^6$  cells and (ii) corrected for plating density to 100%. The results showed that the secretion of TSP by HUVEC decreased at higher cell densities.

#### 7.1.4 Effect of anti-TSP on HUVEC

In order to investigate the role of TSP in the adhesion of endothelial cells to substratum, a HUVEC line was plated at 25% confluent density in the presence of growth medium (3 ml per well) as follows;

medium A:           SFM without EGF

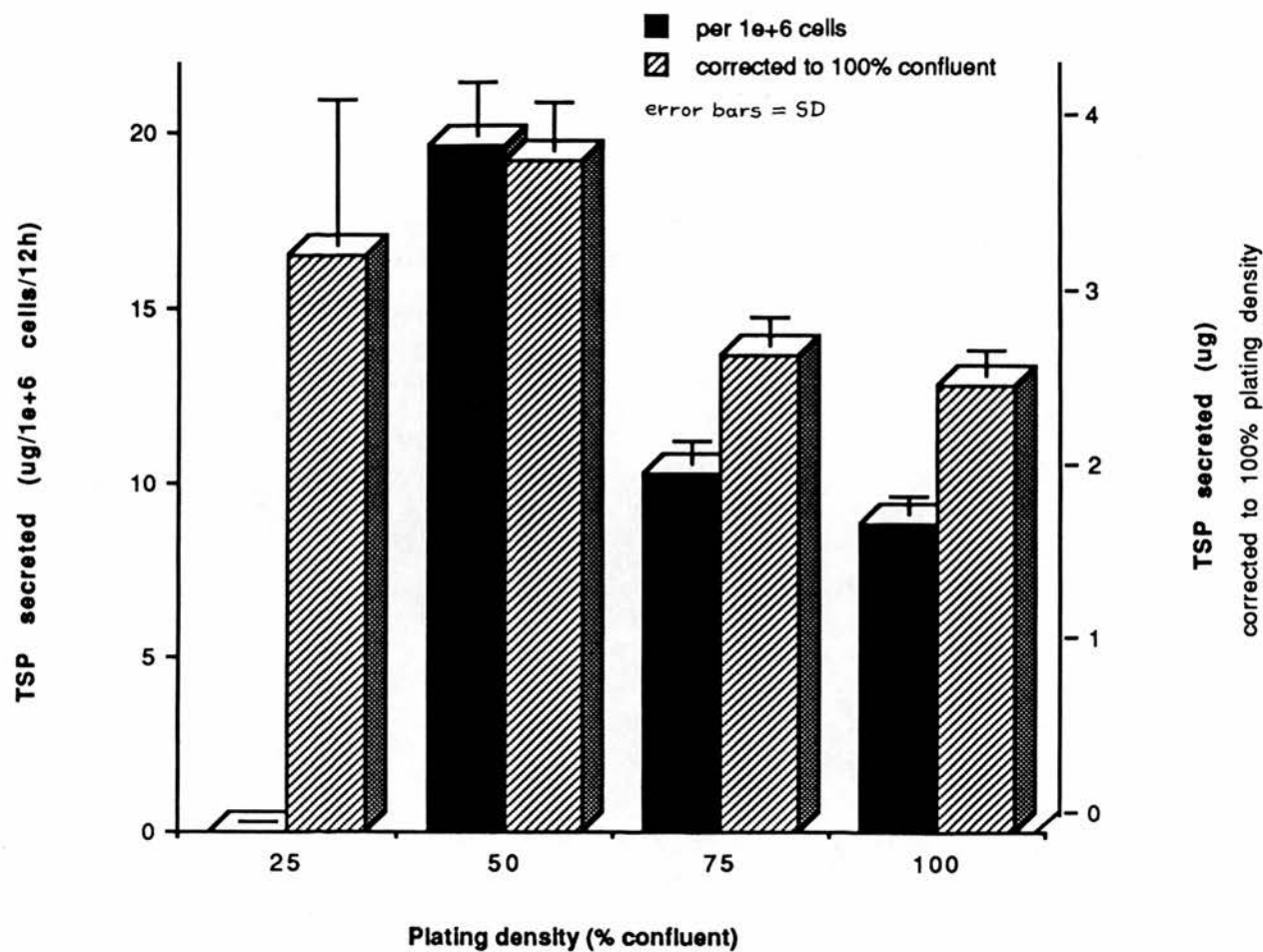
medium B:           medium A + 1% normal rabbit serum

medium C:           medium A + 1% rabbit anti-TSP antiserum

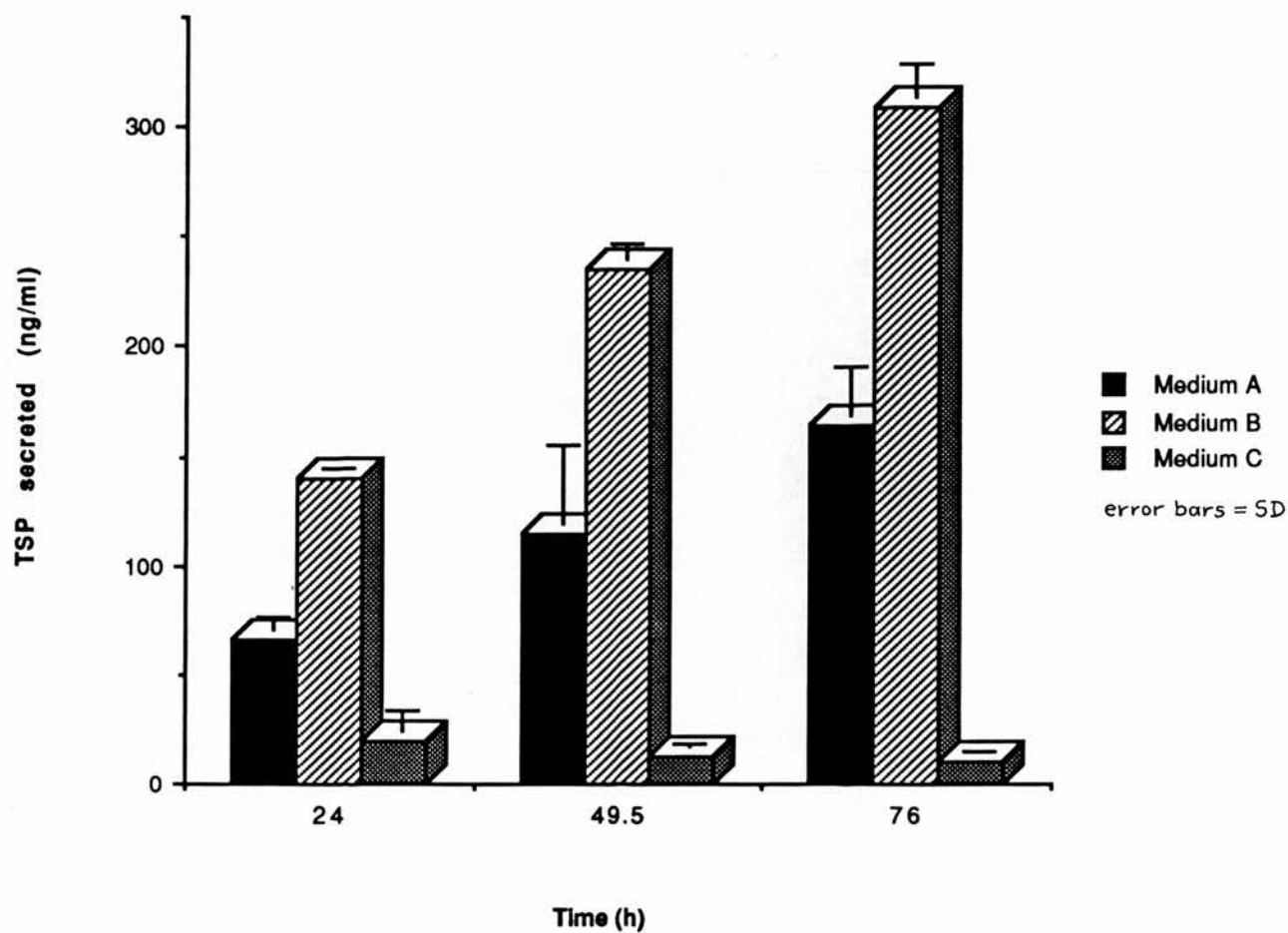
The monolayers were observed by light microscopy for morphological differences and duplicate supernatants were prepared at 24, 50 and 76 h for measurement of TSP as shown in fig.7-3.

After incubation for 24 h, there were no visible morphological differences; each monolayer consisted of cells which were attached and spread. The cell viability in all wells approached 100% (as observed by the ability of cells to exclude the dye Trypan blue). At 50 h, a minority of the cells in medium A were detached from the plate and rounded in shape, whereas the monolayers in media B and C were indistinguishable, with nearly all cells remaining attached

**Fig. 7-2. Effect of cell density on TSP secretion by human umbilical vein endothelial cells (HUVEC)**



**Fig. 7-3. Effect of anti-TSP on TSP secretion by human umbilical vein endothelial cells (HUVEC)**



and spread. At 76 h, many cells in medium A were detached and rounded, while most of the cells in media B and C were still attached and spread, with no apparent difference between the two.

The concentration of TSP measured in wells containing 1% normal rabbit serum was twice that in wells containing SFM alone. Addition of 1% rabbit anti-TSP antiserum was sufficient to bind virtually all the TSP in solution, as shown by the very low level of TSP which could be detected by the radioimmunoassay (10 - 20 ng/ml).

## 7.2 **Fibroblasts**

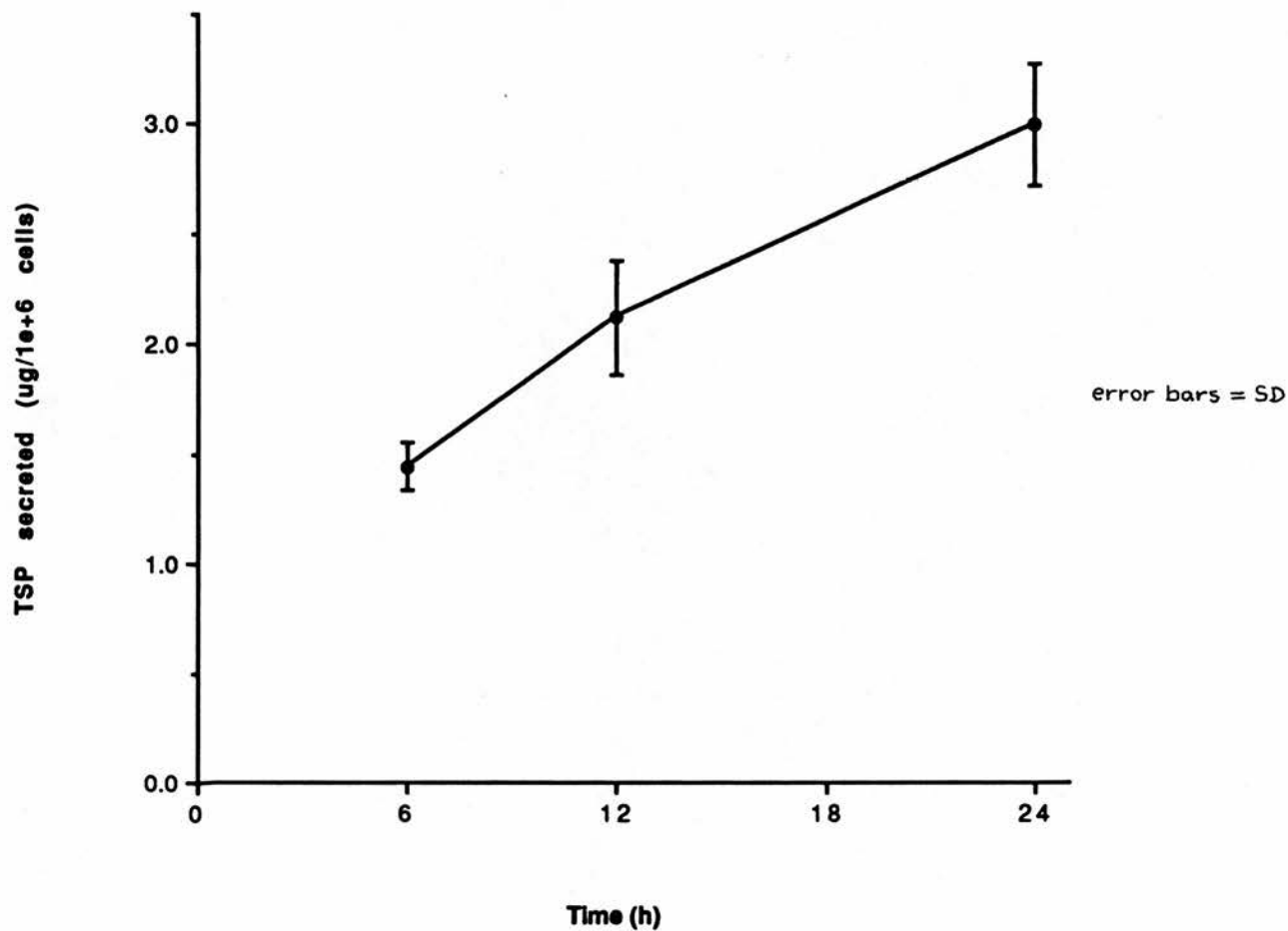
### 7.2.1 **Secretion of TSP by human skin fibroblasts (HSF)**

A normal HSF cell line was incubated in SFM for 6, 12 and 24 h (6 wells for each time point) and measurements of secreted TSP are shown in fig.7-4. Levels of TSP in SFM increased in a linear time-dependent manner and were similar to those previously reported by Hunter et al (1984) for human forearm skin fibroblasts (approximately 3 ug TSP/ $10^6$  cells at 24 h).

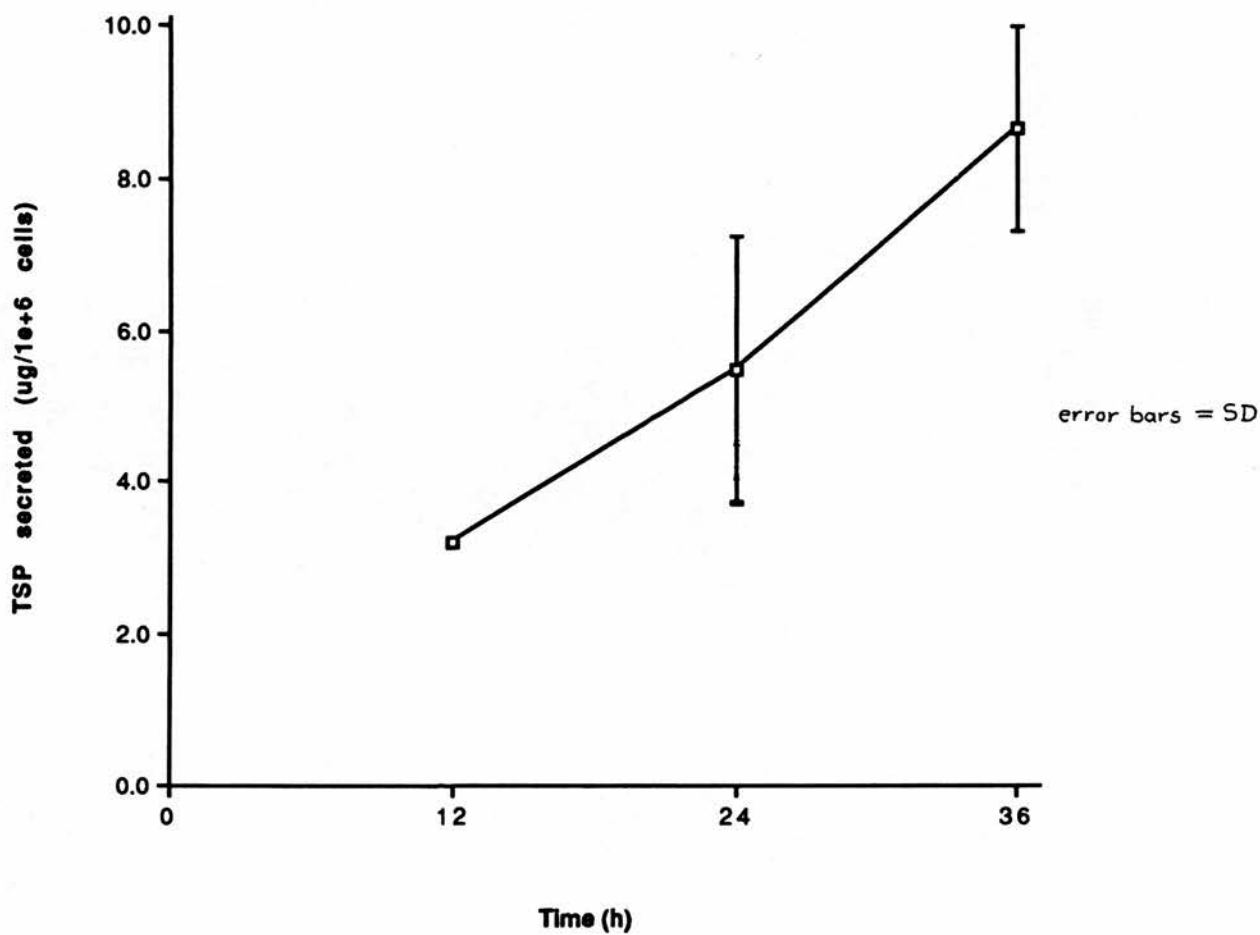
### 7.2.2 **Secretion of TSP by human oral fibroblasts (HOF)**

HOF were incubated in SFM and supernatants removed from duplicate wells at 6, 12 and 24 h for TSP assay. Fig.7-5 shows the secretion of TSP by HOF during this period, which was also linear with time but higher than that observed for HSF (5.5 ug TSP/ $10^6$  cells/24 h for HOF compared with 3.0 ug TSP/ $10^6$  cells/24 h for HSF).

**Fig.7-4. TSP secretion by human skin fibroblasts (HSF)**



**Fig. 7-5. TSP secretion by human oral fibroblasts (HOF)**



### 7.3 MCF-7 Human breast cancer cells

#### 7.3.1 Effect of oestradiol and other hormones on TSP secretion by MCF-7 cells

MCF-7 cells were incubated with SFM containing 0.1% ethanol (control) or 17  $\beta$ -oestradiol ( $10^{-8}$  M) which had been added from a stock ethanolic solution (final ethanol concentration 0.1%). Supernatants were removed from triplicate wells at 24, 51, 76 and 97 h for TSP assay and the results are shown in fig.7-6. The amount of TSP secreted in both cases was relatively low compared with the other cell types previously tested, but also increased in a linear time-dependent manner. Addition of oestradiol caused a small but consistent increase in the secretion of TSP (the difference was only statistically discernible at 97 h).

In another experiment, MCF-7 cells were incubated with a variety of hormones; supernatants were removed from triplicate wells at 24 and 48 h and at the same time the cells were harvested by trypsinisation, washed and resuspended in buffered salt solution for the preparation of cell homogenates using a Microson MS-25 ultrasonic disintegrator (Heat Systems-Ultrasonics Inc., New York, USA). Measurements of TSP in supernatants (secreted TSP) and in homogenates (cell-associated TSP) are shown in fig.7-7. Oestradiol and the two androgens, testosterone and 5 $\alpha$ -dihydrotestosterone, caused small increases in the amounts of secreted and cell-associated TSP. Cortisol caused a decrease in both the secreted and cell-associated TSP after 48 h. The effect of insulin was unusual and quite marked in comparison with the other hormones tested; at both time points a decrease in the amount of TSP

Fig. 7-6. Effect of oestradiol on TSP secretion by MCF-7 human breast cancer cells

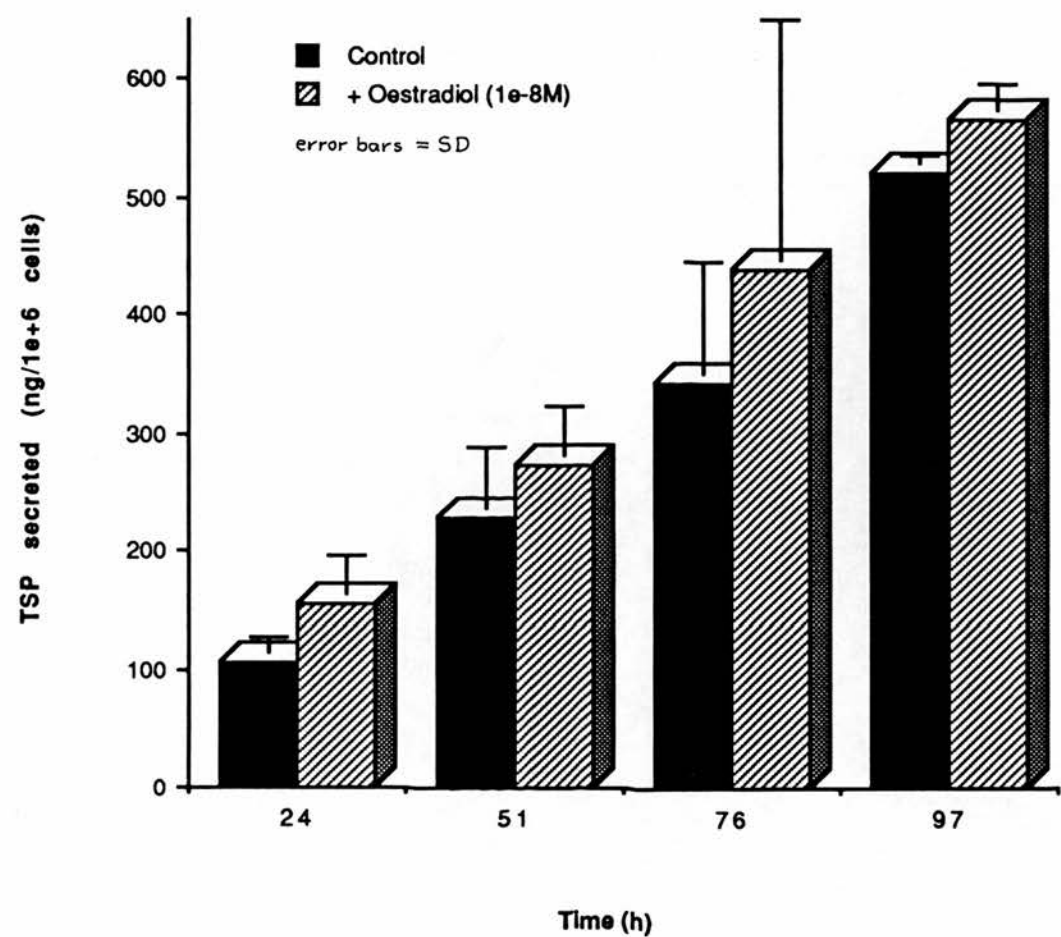




Fig. 7-7a. Effect of hormones on TSP production by MCF-7 human breast cancer cells

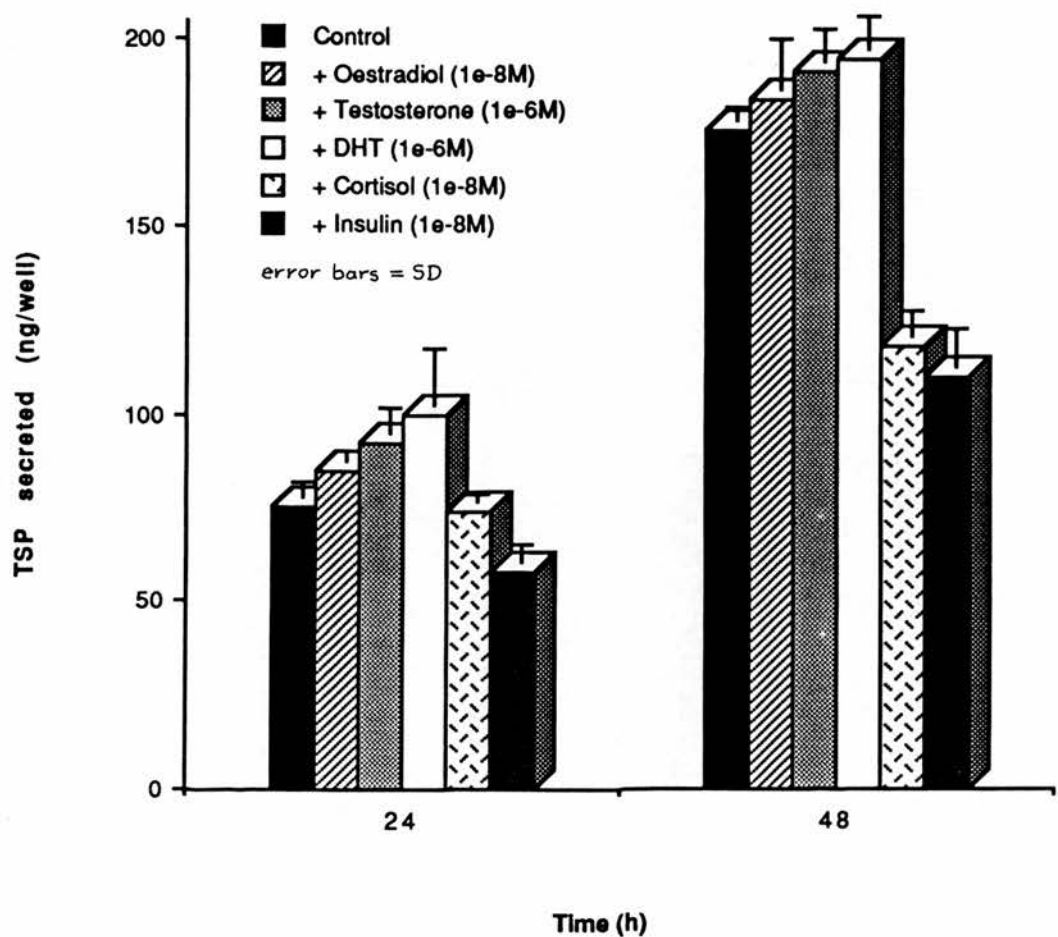
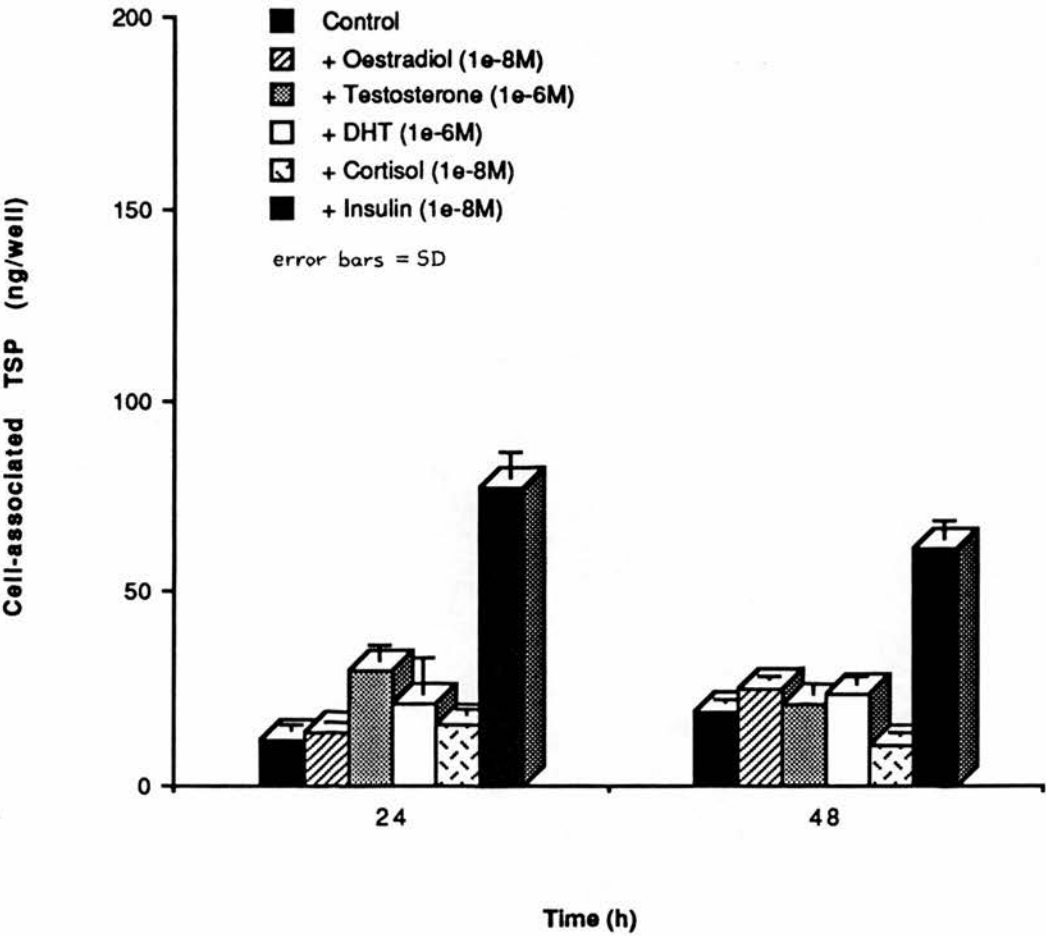


Fig. 7-7b. Effect of hormones on TSP production by MCF-7 human breast cancer cells



secreted was observed, while the corresponding levels in cell homogenates were much higher than in the controls.

### **7.3.2 Effect of EGF on TSP secretion by MCF-7 cells**

MCF-7 cells were incubated in SFM containing 0, 1, or 10 ng/ml EGF. Triplicate supernatants and cell homogenates were prepared at 24 and 48 h as before, and assayed for their TSP content. The results (fig.7-8) showed that addition of EGF had no effect on the production of TSP by MCF-7 cells.

In another experiment, SFM (5 ml) without EGF was replaced every 24 h in triplicate wells over a period of 10 days, followed by SFM containing 10 ng/ml EGF for a further 4 days. The daily production of TSP and cumulative levels are shown in fig.7-9, which clearly illustrates that TSP was secreted, in relatively low amounts, in a linear time-dependent manner, unaffected by the presence of EGF.

### **7.3.3 Comparison of anti-TSP binding curves for MCF-7 SFM and human platelet TSP**

The binding of TSP-like antigen from MCF-7 cells to polyclonal rabbit anti-TSP was compared with that of human platelet TSP by radioimmunoassay (as described in section 2.3). Antigen dilution curves were obtained for a supernatant from MCF-7 cells grown in SFM and for TSP purified from human platelets. The binding curves for both are compared in fig.7-10. Since the two curves were superimposable, it was concluded that TSP-like antigen in MCF-7 SFM was indistinguishable from human platelet TSP by the specific rabbit anti-TSP antiserum used in the radioimmunoassay.

Fig. 7-8. Effect of EGF on TSP production by MCF-7 human breast cancer cells

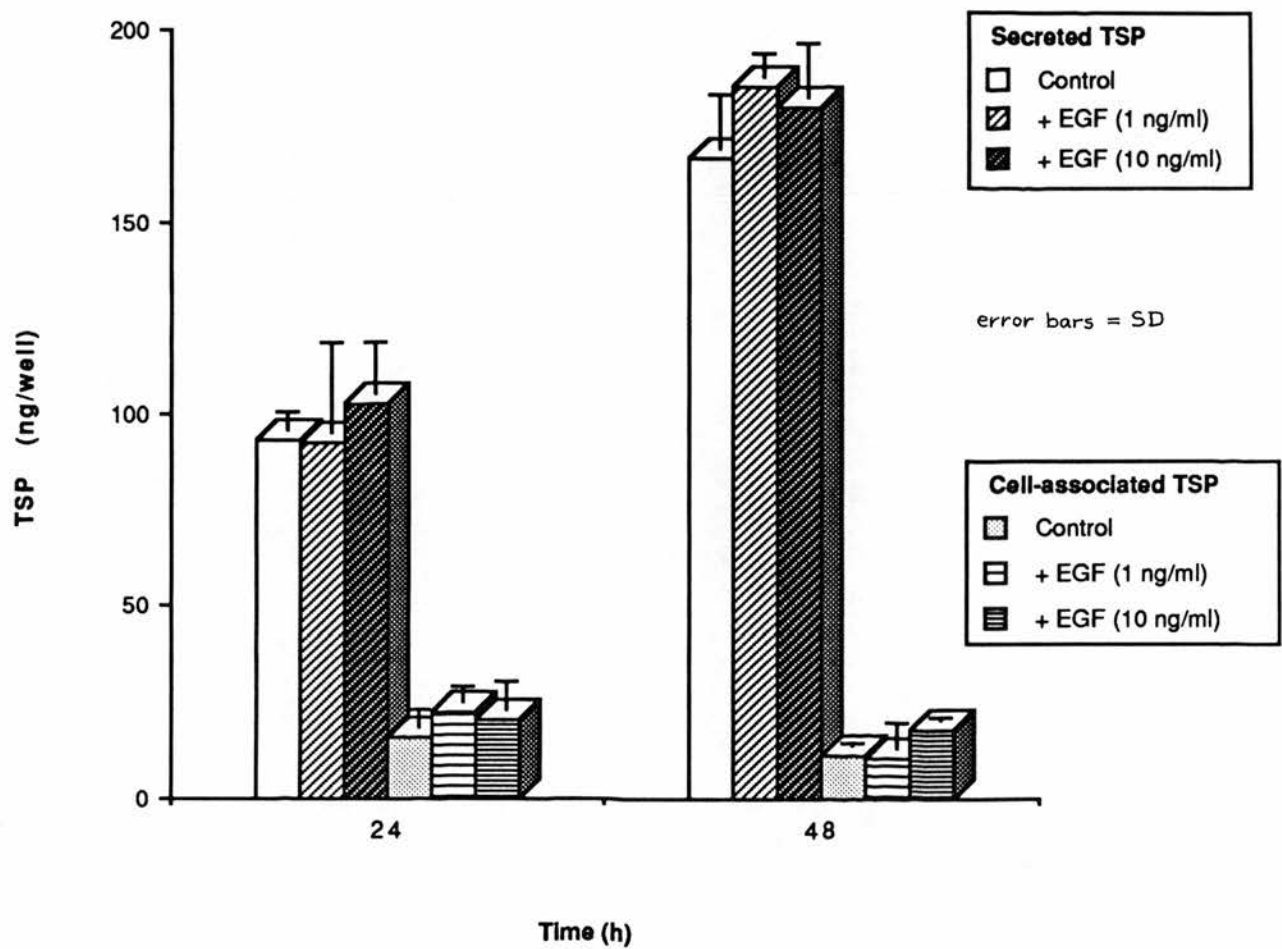


Fig. 7-9. Effect of EGF on daily TSP secretion by MCF-7 human breast cancer cells

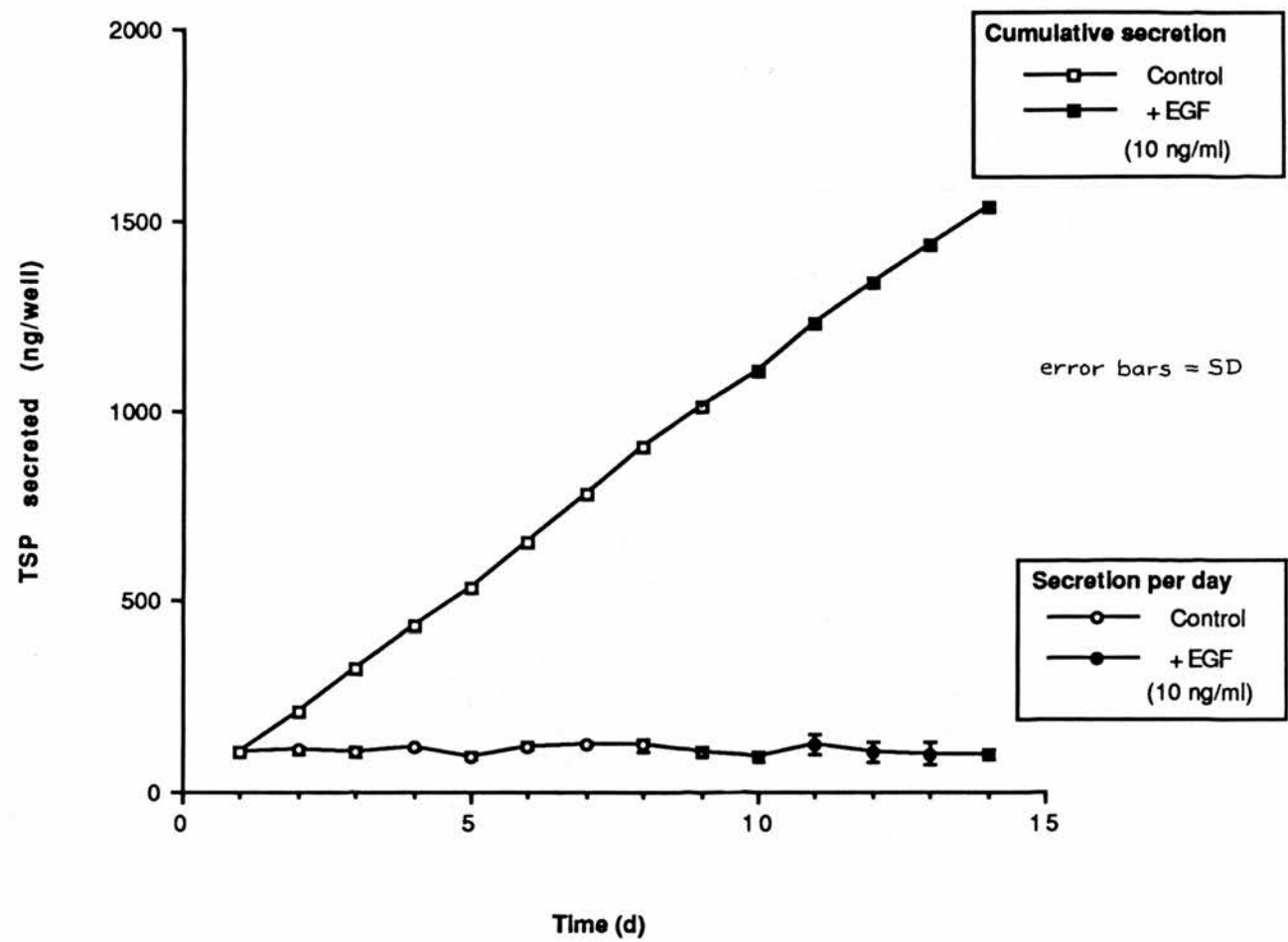
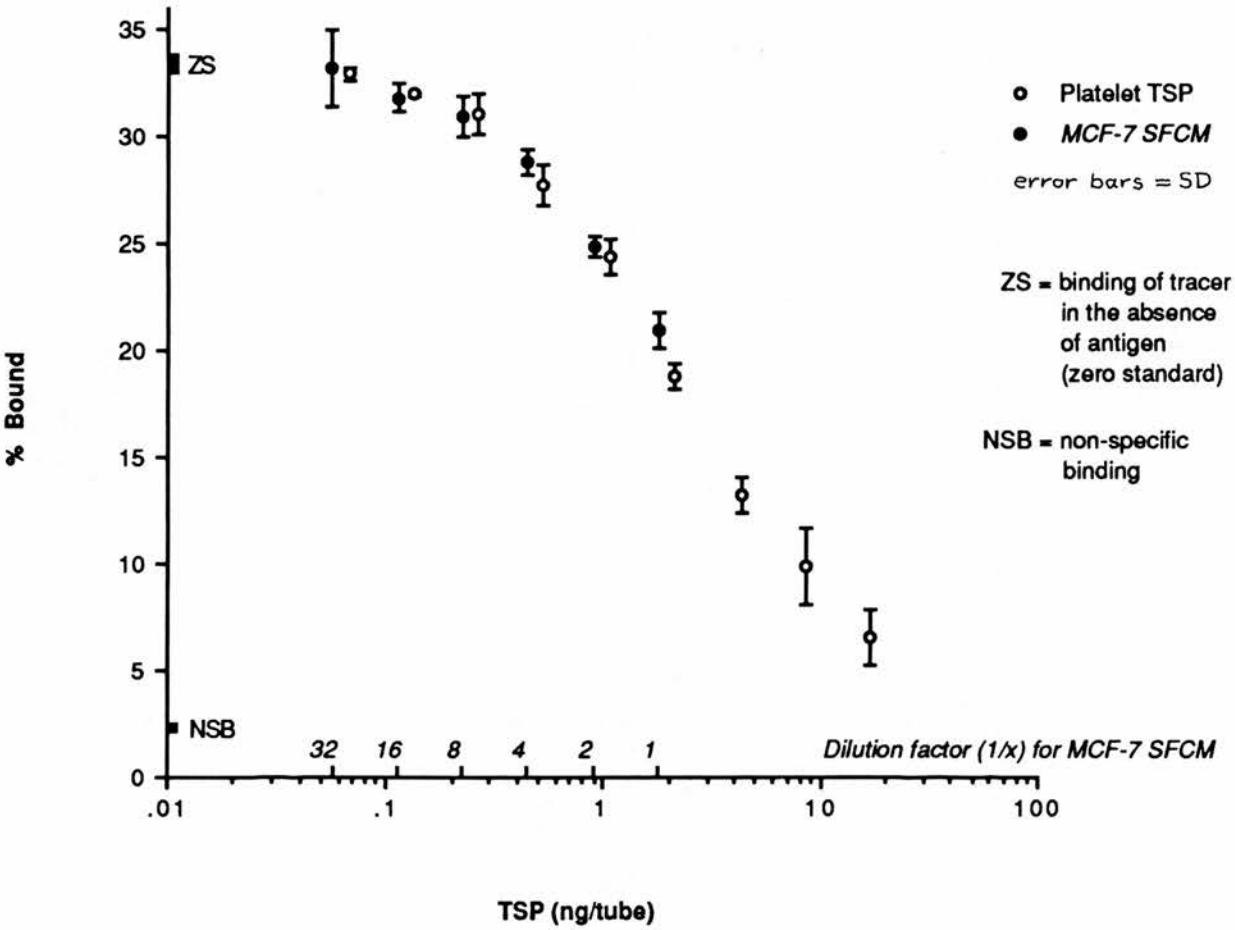


Fig. 7-10. Comparison of antigen dilution curves for human platelet TSP and MCF-7 SFCM by radioimmunoassay



#### 7.4 Discussion

The production of TSP has been demonstrated in a variety of human cells cultured under serum-free conditions. Thus, TSP could be detected by radioimmunoassay in the extracellular medium of cells, irrespective of their type or the tissue source from which they were derived. This is in concordance with the observations of other investigators which show that TSP is synthesised and secreted by a wide range of cells in vitro (section 1.5). Using a panel of monoclonal antibodies, we have previously shown that platelet, endothelial cell and fibroblast TSPs are immunologically identical (Cleazardin et al 1986b) and furthermore, evidence presented here suggests that TSP produced by MCF-7 breast cancer cells is also indistinguishable from platelet TSP using a specific polyclonal antibody. Although TSPs from endothelial cells and fibroblasts appear to be structurally similar, the conformational properties of these forms of TSP may be different from those of platelet TSP (Cleazardin et al 1986b).

The growth conditions (section 2.11) were optimised so that cells could be subcultured over extended periods while still retaining morphological and biochemical properties characteristic of those in the original tissue; for example endothelial cells assumed a "cobblestone" appearance at confluence and synthesised vWF, fibroblasts remained elongated and irregular in shape, MCF-7 cells expressed measurable levels of ER. It is suggested therefore, that in vitro culture of cells may be a useful model system in which to study the contribution of various components of tissues to the overall production of TSP in vivo, and the factors which might influence such production.

Although TSP was found in all the cell cultures under study, quantitative differences were observed between each cell type. In general, high levels of TSP were secreted by endothelial cells and fibroblasts whereas much lower levels were measured in the extracellular medium of MCF-7 cells. In all cases however, the secretion of TSP occurred in a continuous, almost linear manner under the serum-free culture conditions.

The production of TSP has been most extensively studied in endothelial cells; the data presented here are comparable with the findings of other groups, and fall within the range of levels of secreted TSP which have been reported previously (table 7-1). The relatively large variation in the amounts of TSP detected in endothelial cell cultures probably reflects the different species and tissues from which they have been derived, the varying composition of the growth media used, and the different sensitivities and specificities of the TSP assays.

It is clear that several factors influence the amounts of TSP detected; of importance is the presence of serum which contains variable levels of proteins (including TSP and immunoglobulins) which may interfere with TSP assays, and growth factors and hormones which can have potent effects on the growth and metabolism of cells. Unlike most of the studies reported by others, the present investigations have been performed using chemically defined serum-free media in order to eliminate unknown variables in the growth conditions due to serum and to allow comparisons to be made between different cell types cultured under standardised conditions.

Although other groups have studied endothelial cells isolated



Table 7-1. Secretion of TSP by endothelial cells

Tissue source	Growth medium	Secreted TSP	Ref.
Bovine aorta	Weymouth's + 1% porcine serum	1.0 ug/10 <sup>6</sup> cells/18 h	(1)
Human umbilical cord vein	Medium 199 + 20% rabbit serum	49 ug/10 <sup>6</sup> cells/24 h	(2)
Human umbilical cord vein	MEM + 20% rabbit serum	21 ug/10 <sup>6</sup> cells/24 h	(3)
Human umbilical cord vein	Serum-free conditioning medium	8.7 ug/10 <sup>6</sup> cells/24 h	(4)

References:

- 1) Raugi et al 1982
- 2) Mosher et al 1982
- 3) Jaffe et al 1983
- 4) Hunter et al 1984

from foetal and adult tissue, and from arteries and veins, no direct comparisons between the secretion of TSP by these cells were made. It is therefore of interest that differences have been found in the levels of TSP secreted by human endothelial cells derived from foetal (HUVEC) and adult (HSVEC) veins, and between those from umbilical cord artery (HUAEC) and vein (HUVEC). While it is not clear to what extent these findings might reflect the situation in vivo, it may be concluded that the differences observed are due to characteristics of the cells themselves, since each cell type was grown under identical conditions. The higher levels of TSP found in the extracellular medium of HUVEC compared with HSVEC might be evidence that the expression of TSP varies according to the stage of development of a tissue. Kramer et al (1985) showed that TSP was a significant component of the subendothelial matrix of neonatal microvascular endothelial cells whereas those cultured from adult skin produced a matrix containing negligible amounts of TSP, while Murphy-Ullrich et al (1987a) observed an extensive distribution of TSP in sections of human foetal limb using immunohistochemical techniques but were unable to localise TSP in various human adult tissues. Taken together, these results may support the hypothesis that the production of TSP by cells, and its incorporation into their extracellular matrices, is higher during certain stages of development when TSP may be involved in tissue reorganisation and cell migration (section 1.4.4).

Endothelial cells form a smooth, non-thrombogenic lining of both arteries and veins, but the structures of these two types of vessel are markedly different. Arteries have a thick wall containing elastic and collagen fibres and smooth muscle, which enables them

to withstand the pulsed flow of blood under high pressure from the heart. On the other hand, veins carry blood at low pressure and possess relatively thin, non-elastic walls. The finding that HUAEC produce approximately twice as much TSP in culture as their venous counterparts is interesting and could relate to the structural and functional differences of arteries and veins, since TSP has been localised around small blood vessels and along the luminal surface of aorta in situ (Wight et al 1985).

Increasing cell density caused a marked reduction in the secretion of TSP by endothelial cells. This is in agreement with the studies of Mumby et al (1984) who observed that the production of TSP by endothelial cells, fibroblasts and smooth muscle cells was density-dependent. They concluded that the higher level of TSP in the extracellular medium of low-density cells was due to increased secretion rather than decreased degradation or its incorporation into matrix. In contrast, cell density did not modulate the secretion of two other matrix proteins, fibronectin and collagen, by fibroblasts (Mumby et al 1984). In endothelial cells, TSP and fibronectin secretion rates appear to depend solely on the rate of de novo protein synthesis, unlike another adhesive protein, vWF, which is stored in secretory organelles (Reinders et al 1985). Thus, the synthesis and secretion of different matrix components may be under separate control mechanisms. The increased production of TSP by cells at low density may be evidence of a role for TSP at sites of injury and wound healing where cell-cell contacts are reduced, since this protein has been found to be specifically associated with clots formed in situ (Murphy-Ullrich et al 1985) and atherosclerotic plaques (Wight et al 1985).

Adhesion of endothelial cells to fibronectin-coated plastic was not visibly affected by the presence of anti-TSP antiserum in sufficient quantity to bind all the TSP secreted into the extracellular medium by these cells. Although TSP has been shown to promote the cell-substratum adhesion of a variety of human cells (Lawler et al 1988, Tuszynski et al 1987b, Varani et al 1986, Roberts et al 1987, Roberts et al 1985), there is conflicting evidence concerning the effect of TSP on endothelial cell adhesion (Lahav 1988, Murphy-Ullrich & Hook 1989). In reports where TSP has been shown to mediate cell adhesion, this activity could be blocked by synthetic peptides containing the RGD sequence and by certain anti-TSP antibodies, suggesting that one or more specific domains of TSP may be involved in cell-substratum interactions. The lack of inhibition of endothelial cell adhesion by the rabbit polyclonal antibody used here could indicate that TSP is not important for the attachment and spreading of endothelial cells on fibronectin, or that this antibody is not specifically directed against the region of the TSP molecule which is active in cell adhesion. There was evidence to suggest that a serum factor other than TSP was necessary for the maintenance of adhesion and normal morphology, since most cells plated in SFM alone became rounded and detached from the substratum after 76 h, whereas those plated in the presence of 1% serum remained attached and spread.

Like endothelial cells, fibroblasts synthesise and secrete relatively large amounts of TSP and the data presented here are comparable with those reported by others (table 7-2). The variation in amounts of fibroblast-secreted TSP which have been detected is probably mainly due to differences in the culture conditions and

Table 7-2. Secretion of TSP by fibroblasts

Tissue source	Growth medium	Secreted TSP	Ref.
Human foreskin	Weymouth's + 1% porcine serum	2.5 ug/10 <sup>6</sup> cells/18 h	(1)
Human foreskin	MEM + 20% rabbit serum	15.7 ug/10 <sup>6</sup> cells/24 h	(2)
Human foetal lung	MEM + 20% rabbit serum	5.8 ug/10 <sup>6</sup> cells/24 h	(2)
Human foreskin	DMEM + 5% rabbit serum	0.36 ug/10 <sup>6</sup> cells/24 h	(3)
Human foreskin	Weymouth's + 5% rabbit serum	3.0 ug/10 <sup>6</sup> cells/24 h	(3)

References:

- 1) Raugi et al 1982
- 2) Jaffe et al 1983
- 3) Mumby et al 1984

assay procedures employed, although the present results show that the tissue source may also be a factor which determines the quantity of TSP produced.

MCF-7 breast cancer cells produced much smaller but measurable amounts of TSP in a continuous, linear manner. Cumulative secretion over a period of 14 days in serum-free culture confirmed that the TSP detected in extracellular medium was of MCF-7 cell origin rather than due to leaching of any TSP adsorbed from the foetal calf serum used in the initial growth medium. These results represent the first quantitation of TSP secretion by cells of epithelial origin and therefore indicate that the production of TSP is more ubiquitous than has previously been known. Such secretion is unlikely to fully account for the high levels of TSP found in breast cancer (section 6.1) but may at least contribute to the TSP content of that tissue.

Since the growth and metabolism of MCF-7 cells is known to be modulated by hormones and other factors through binding to specific cellular receptors, it was of interest to investigate the effects of these agents on TSP production by MCF-7 cells. Given that the secretion of TSP by endothelial cells appears to depend solely on the rate of de novo protein synthesis (Reinders et al 1985), it might be expected that agents which modulate the latter should also affect the rate of TSP production in these and perhaps other cell types. The steroid hormones oestradiol, testosterone and dihydrotestosterone all caused small increases in TSP secretion by MCF-7 cells at concentrations which have previously been shown to stimulate macromolecular synthesis and proliferation of these cells (Lippman et al 1976a,b). Similarly, the reduction in TSP secretion

observed in the presence of cortisol was consistent with previously reported inhibitory effects of glucocorticoids on the growth of MCF-7 cells (Lippman et al 1976c). MCF-7 cells have been shown to be strikingly sensitive to insulin (Osborne et al 1978) and, indeed, insulin had a marked effect on TSP production. While the amount of secreted TSP was much lower in the presence of insulin, that which was associated with the cells themselves increased considerably, suggesting that insulin may inhibit the transport of TSP into the extracellular medium by MCF-7 cells.

It has been reported that breast cancer cells produce, and possess receptors for, several growth factors including EGF, which may stimulate their growth and mediate some of the effects of hormones in an autocrine or paracrine manner (Lippman et al 1986). The present studies showed that EGF had no effect on TSP production by MCF-7 cells. The modulation of the latter by oestradiol and other hormones which has been observed was therefore either a direct hormone effect or was mediated by factors other than EGF.

## CHAPTER EIGHT

### PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST THROMBOSPONDIN

In order to facilitate studies of TSP, monoclonal antibodies (MAbs) were raised to this macromolecule which would potentially be more powerful research tools than the existing polyclonal rabbit anti-TSP antiserum. Purified human platelet TSP was used as immunogen for the generation of murine MAbs in an attempt to produce a panel of antibodies having a range of TSP-binding specificities, which could be used to investigate the activities of this multifunctional glycoprotein. Methods for bulk production and purification of murine anti-TSP MAbs were developed, and a preliminary study of the binding activities of the resulting antibody panel was done using two different assay systems, ELISA (section 2.8) and SIBA (section 2.9).

The production of monoclonal antibodies against human platelet TSP was carried out in collaboration with Dr L. Micklem, Department of Surgery, Edinburgh (see section 2.12 for methods).

#### 8.1 Immunisation of mice with TSP, hybridisation and production of monoclonal antibodies

Immunisations of mice were carried out on three separate occasions as follows. Five mice were immunised with human platelet TSP prepared in the presence of  $\text{Ca}^{2+}$  and 6+5 mice immunised with TSP prepared in the presence of EDTA. None (0%) of the mice which



received  $\text{Ca}^{2+}$ -replete TSP produced a detectable antibody response, whereas six (55%) of those which received  $\text{Ca}^{2+}$ -deplete TSP showed positive responses when their sera were tested for  $^{125}\text{I}$ -TSP binding in a modified RIA.

Spleen cells from three responding mice were used in hybridisations and yielded 10 hybridoma cell lines which were positive for antibody secretion by anti-TSP screening ELISA, from a total of 329 clones tested. The isotypes and titres of the monoclonal antibodies produced in bulk by these cell lines are shown in table 8-1.

## **8.2 Bulk production of mouse monoclonal anti-TSP antibody by continuous stirred culture**

One clone of cells producing anti-TSP IgM antibodies was grown in a CelliGen cell culture system. The cell growth curve and anti-TSP levels over 11 days of continuous stirred culture are shown in fig.8-1. Production of anti-TSP antibody closely followed cell growth. During a period of accidentally-induced anoxia in the middle of the 11-day period, production of anti-TSP activity was seen to level off. This was then followed by a sharp decrease in cell viability and anti-TSP activity.

## **8.3 Purification of mouse monoclonal IgG by affinity chromatography on protein A**

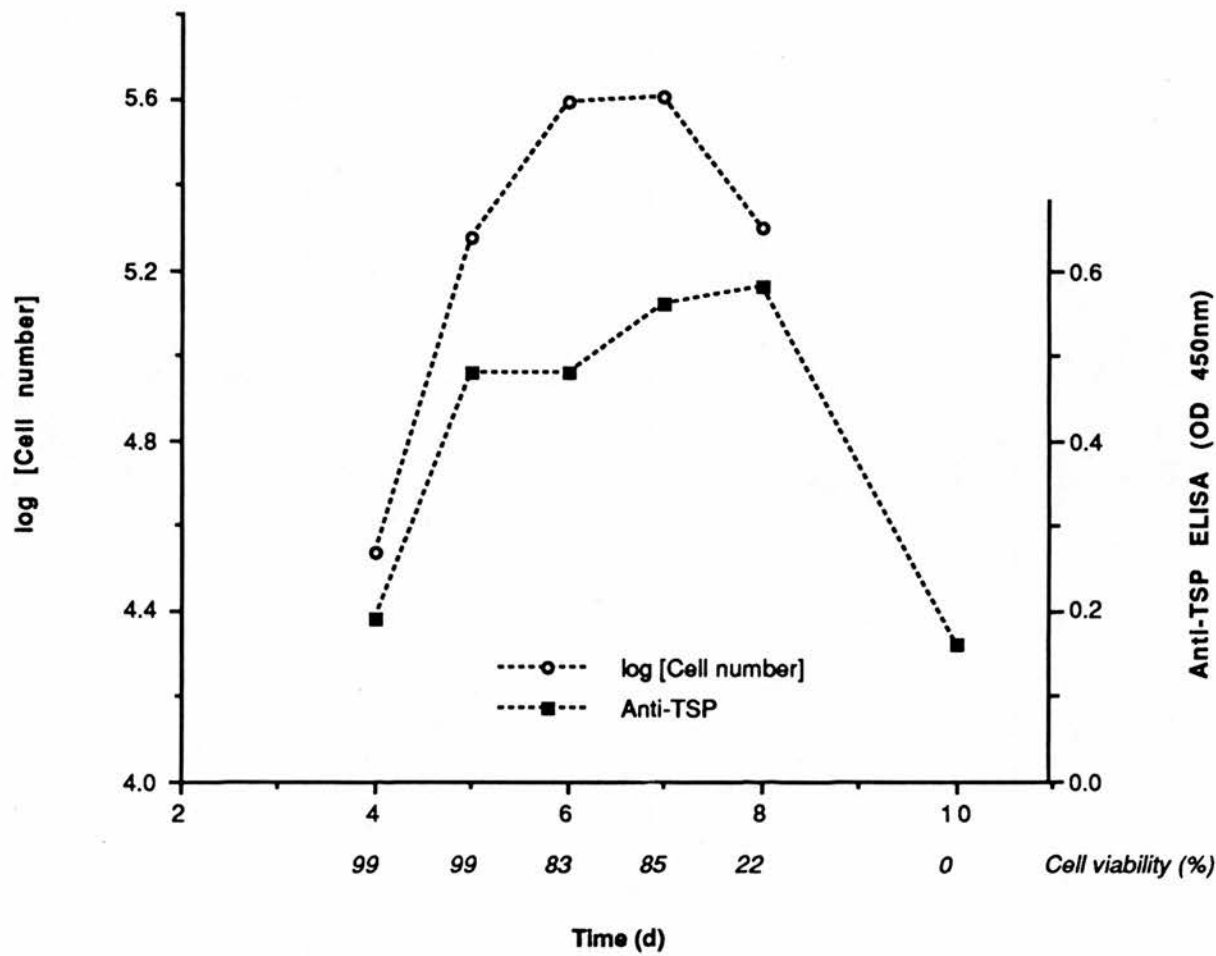
The elution profile for a mouse monoclonal IgG<sub>1</sub> anti-TSP antibody is shown in fig.8-2. Concentrated bulk culture supernatant was loaded onto a protein A-Sepharose 4B column and eluted as described in section 2.12.6. Antibody which bound to the column,

Table 8-1. Murine monoclonal antibodies produced against human platelet TSP

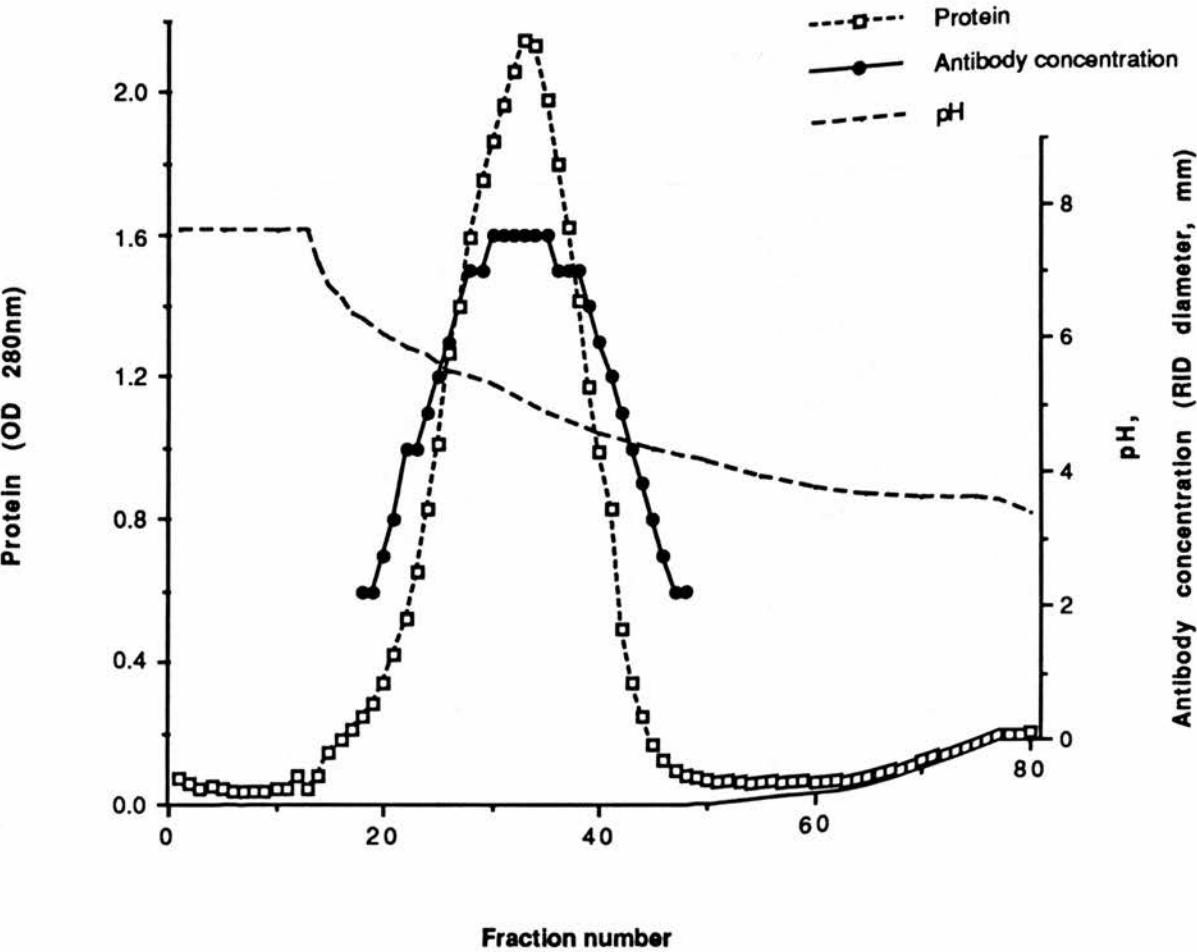
Antibody	Type	Titre* of bulk culture medium	Titre* of ascitic fluid
ESTs3	IgM	$10^0$	$10^3$
ESTs4	IgM	$10^0$	$10^4$
ESTs6	IgM	$10^0$	$10^3$
ESTs8	IgM	$10^0$	$10^4$
F189/2.1	IgM	-	$10^5$
F189/12.3	IgG <sub>1</sub>	$10^1$	$> 10^5$
F189/20.4	IgM	$10^0$	$10^4$
F189/53.2	IgG <sub>1</sub>	$> 10^4$	$> 10^5$
F189/58.1	IgM	$10^0$	$10^3$
F189/76.2	IgM	$10^0$	$10^5$

\* Highest dilution factor which gave a positive result by ELISA (section 2.8)

**Fig. 8-1. Production of murine monoclonal antibody (ESTs8) by hybridoma cells  
In continuous stirred culture**



**Fig. 8-2. Purification of murine monoclonal IgG anti-TSP antibody (F189/53.2) by affinity chromatography on protein A-Sepharose 4B**



and which was separated from other proteins in the culture supernatant at high pH, was eluted in a peak between pH 6.5 - 5.5.

#### **8.4 Purification of mouse monoclonal IgM by gel filtration on Sephacryl S-300**

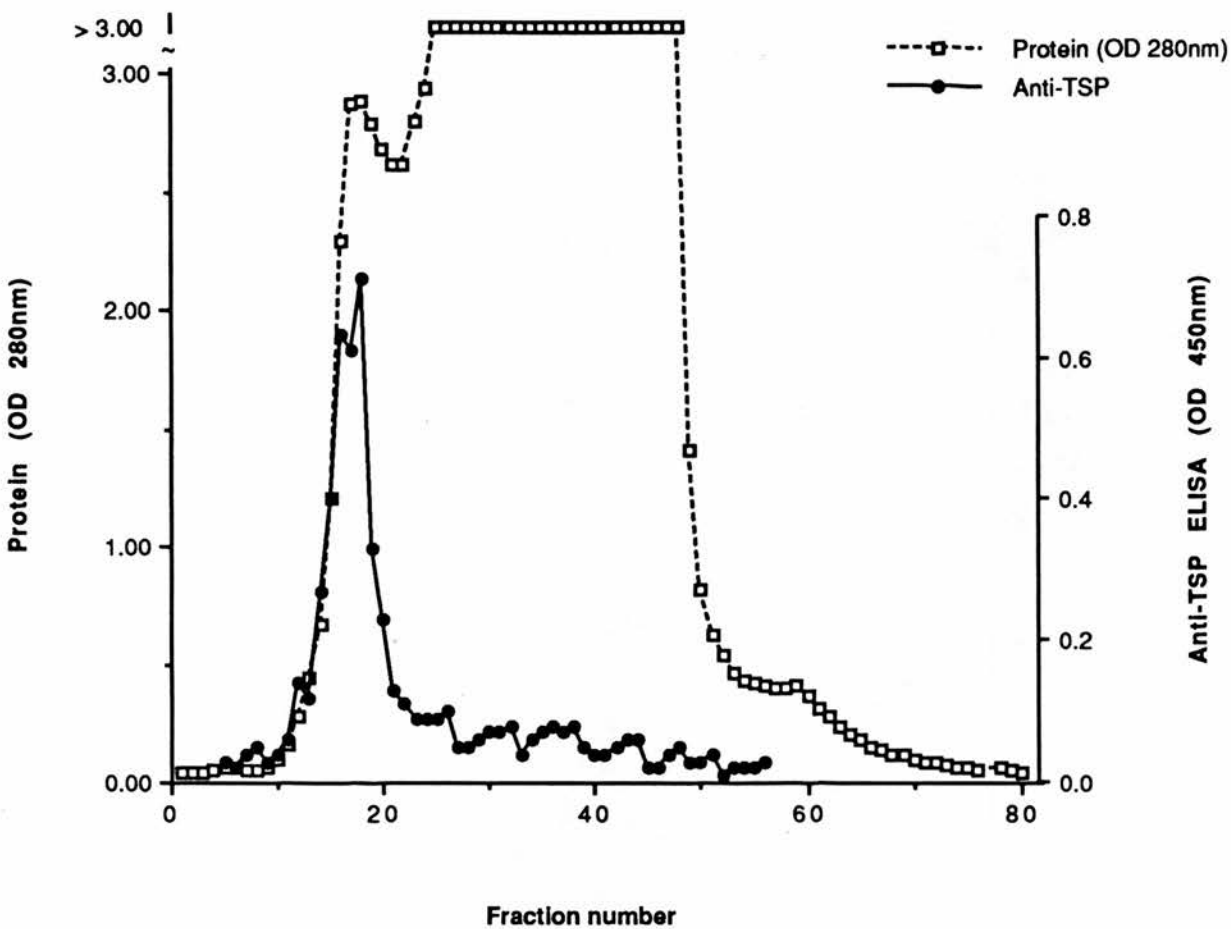
The elution profile for a mouse monoclonal IgM anti-TSP antibody is shown in fig.8-3. Concentrated bulk culture supernatant was loaded onto a Sephacryl S-300 column and eluted as described in section 2.12.8. The high molecular weight IgM antibody was separated from smaller proteins in the culture supernatant, and was eluted close to the void volume of the column.

#### **8.5 Binding patterns of mouse monoclonal anti-TSP antibodies to platelet proteins in an enzyme-linked immunosorbant assay (ELISA)**

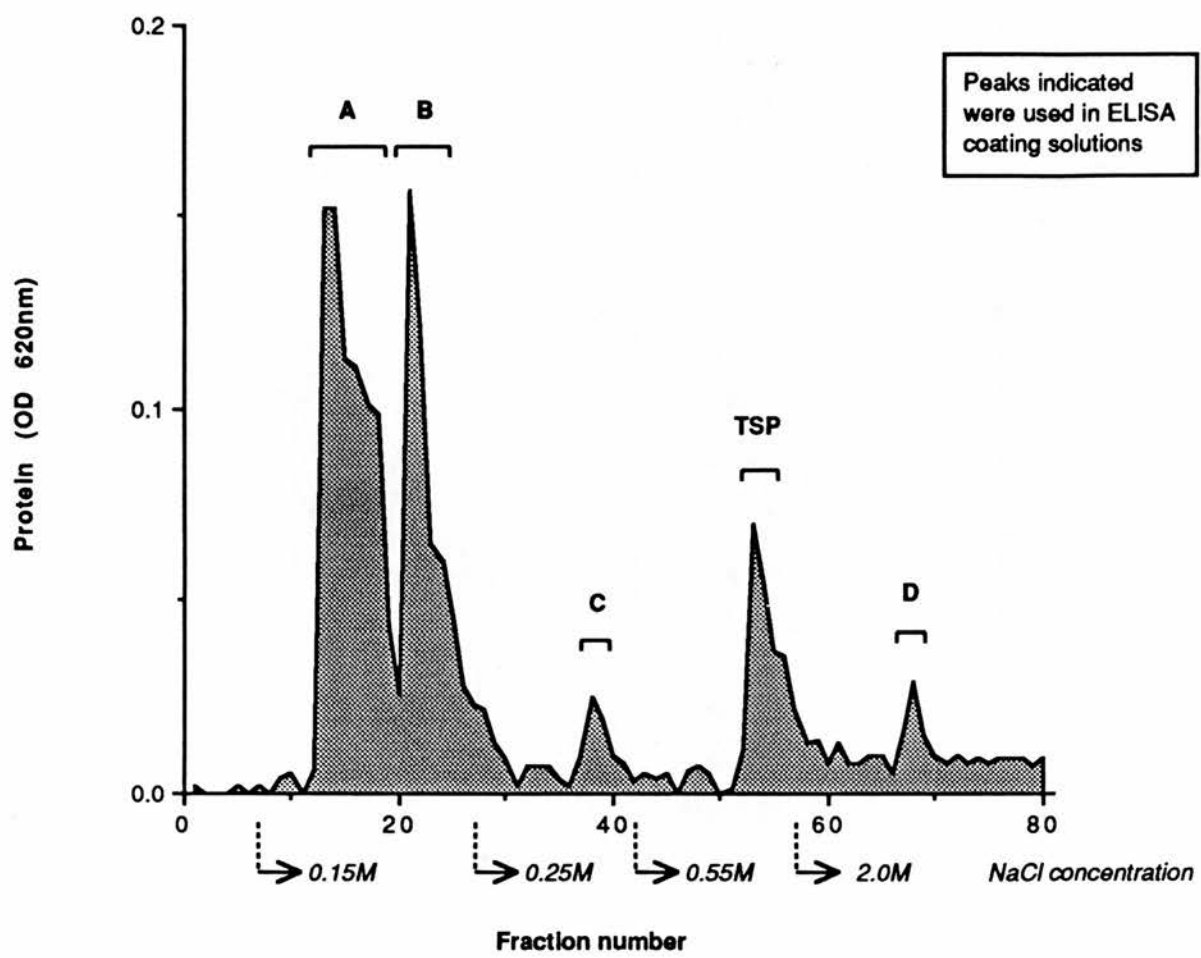
The ELISA (section 2.8) which was used in the screening of clones for anti-TSP production, was modified by substituting other peaks from the separation of thrombin-stimulated platelet supernatant using heparin affinity chromatography, for TSP in the coating step. Thus, the ELISA coating solutions A - D corresponded to the peaks A - D in the elution profile shown in fig.8-4. Each peak was diluted in 15 mM Tris-HCl, 2 mM  $\text{CaCl}_2$ , 0.02%  $\text{NaN}_3$ , pH 7.6, to a protein concentration of 10 ug/ml before use.

The following mouse monoclonal anti-TSP antibodies were tested using this ELISA; ESTs3, ESTs4, ESTs6, ESTs8, F189/2.1, F189/12.3, F189/20.4, F189/53.2, F189/58.1, F189/76.2 (all as undiluted culture supernatants), MA-I and MA-II (1/500 dilutions of purified antibody solutions, 6.3 mg/ml and 7.7 mg/ml respectively; generous

**Fig. 8-3. Purification of murine monoclonal IgM anti-TSP antibody (F189/20.4) by gel filtration on Sephacryl S-300**



**Fig. 8-4. Heparin-Sepharose affinity chromatogram of thrombin-stimulated human platelet releasate**



gifts from Dr J. Lawler, Boston, USA). Antibodies MA-I and MA-II have already been well characterised (Lawler et al 1985).

Results of binding of the monoclonal anti-TSP antibodies to the various platelet protein peaks in the ELISA are summarised in table 8-2. In general, antibodies of the IgG<sub>1</sub> subclass (F189/12.3, F189/53.2, MA-I and MA-II) showed much higher binding to TSP than those of the IgM type, two of which (ESTs3 and ESTs8) were negative. An exception to this was F189/76.2 (IgM-type) which gave the strongest binding to all the platelet protein peaks. A wide range of binding patterns to coating solutions A - D was observed, each of which was characteristic of the particular antibody used.

#### **8.6 Binding of mouse monoclonal anti-TSP antibodies to human platelet TSP, proteins from breast cyst fluid, cultured cells and a variety of animal sera in a slot-immunobinding assay (SIBA)**

The following mouse monoclonal anti-TSP antibodies were tested for binding various antigens in a SIBA (section 2.9); ESTs3, ESTs4, ESTs6, ESTs8, F189/2.1, F189/20.4, F189/58.1, F189/76.2 (all 1/100 dilutions of ascites), F189/12.3, F189/53.2 (1/1000 dilutions of antibody solutions, approximately 1 mg/ml, purified from culture supernatants) MA-I, MA-II (1/1000 dilutions of purified antibody solutions, 6.3 mg/ml and 7.7 mg/ml respectively), FTI 10, FTI 52, FTI 53 (1/1000 dilutions of ascites; produced in collaboration with Prof N. Crawford, Royal College of Surgeons, London).

The antigen preparations (100 ul/slot) used were; human platelet TSP (diluted 1/20 - 1/20000 in TBS; 320 - 0.32 ng TSP by RIA), human breast cyst fluid (1/100, 48 ng TSP), normal human serum (1/100, 17 ng TSP), various animal sera (all at 1/100), serum-free



Table 8-2. Binding patterns of monoclonal anti-TSP antibodies to platelet proteins in an ELISA

Antibody	TSP	ELISA coating solution			
		'A'	'B'	'C'	'D'
ESTs3	-	++	+++	+	-
ESTs4	+	+	+	+	-
ESTs6	+	+	+	-	-
ESTs8	-	+	+	+	-
2.1	+	-	+++	+++	-
12.3	+++	-	-	-	++
20.4	+	-	-	-	-
53.2	+++	+	+++	+++	+++
58.1	+	-	-	++	-
76.2	+++	+++	+++	+++	+++
MA-I	+++	+	++	+++	+++
MA-II	+++	-	-	-	-

Data from several experiments.

Key:        - = < 0.2 (OD units; 450nm)  
          + = 0.2 - 0.8  
          ++ = 0.8 - 1.4  
          +++ = > 1.4

conditioned media from human umbilical artery endothelial cells (1/2, 70 ng TSP; HUAEC SFCM), human oral fibroblasts (undiluted, 81 ng TSP; HOF SFCM) and MCF-7 human breast cancer cells (undiluted, 1 ng TSP; MCF-7 SFCM).

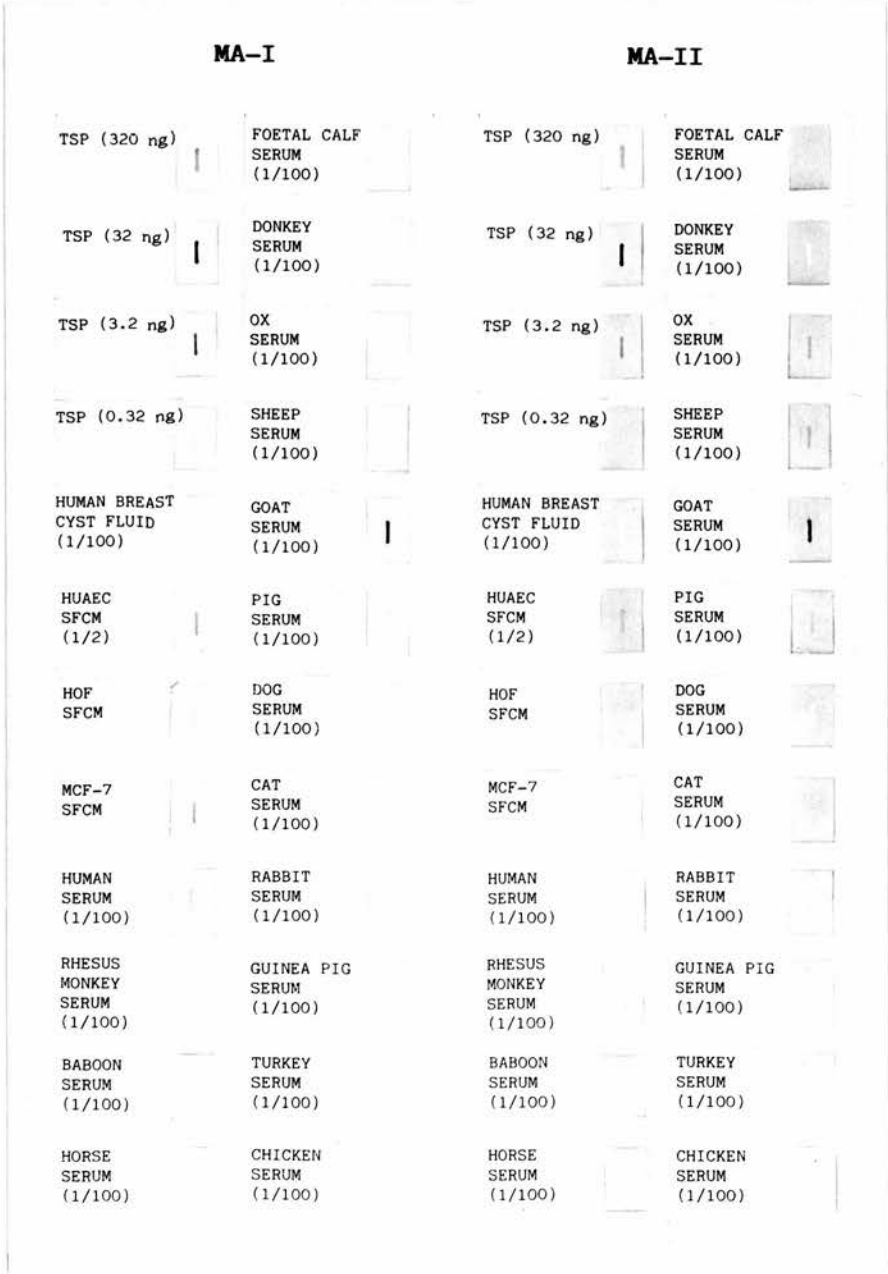
Results of the SIBA are shown in figs.8-5a - 8-5g. Again, a wide range of binding patterns was observed as was the case by ELISA (section 8.5).

Antibodies which bound strongly to TSP in the ELISA (MA-I, MA-II, F189/12.3, F189/53.2, F189/76.2) also showed high affinities for TSP in the SIBA, whilst those which did not bind TSP in the ELISA (ESTs3 and ESTs8) were also negative by SIBA, even at the highest TSP loading. It was interesting that ESTs3 and ESTs8 caused high non-specific binding to the Immobilon PVDF membrane and this was not observed with any of the other antibodies tested. ESTs6 was the only antibody which showed a marked difference in TSP-binding in the SIBA (strong) compared with the ELISA (weak).

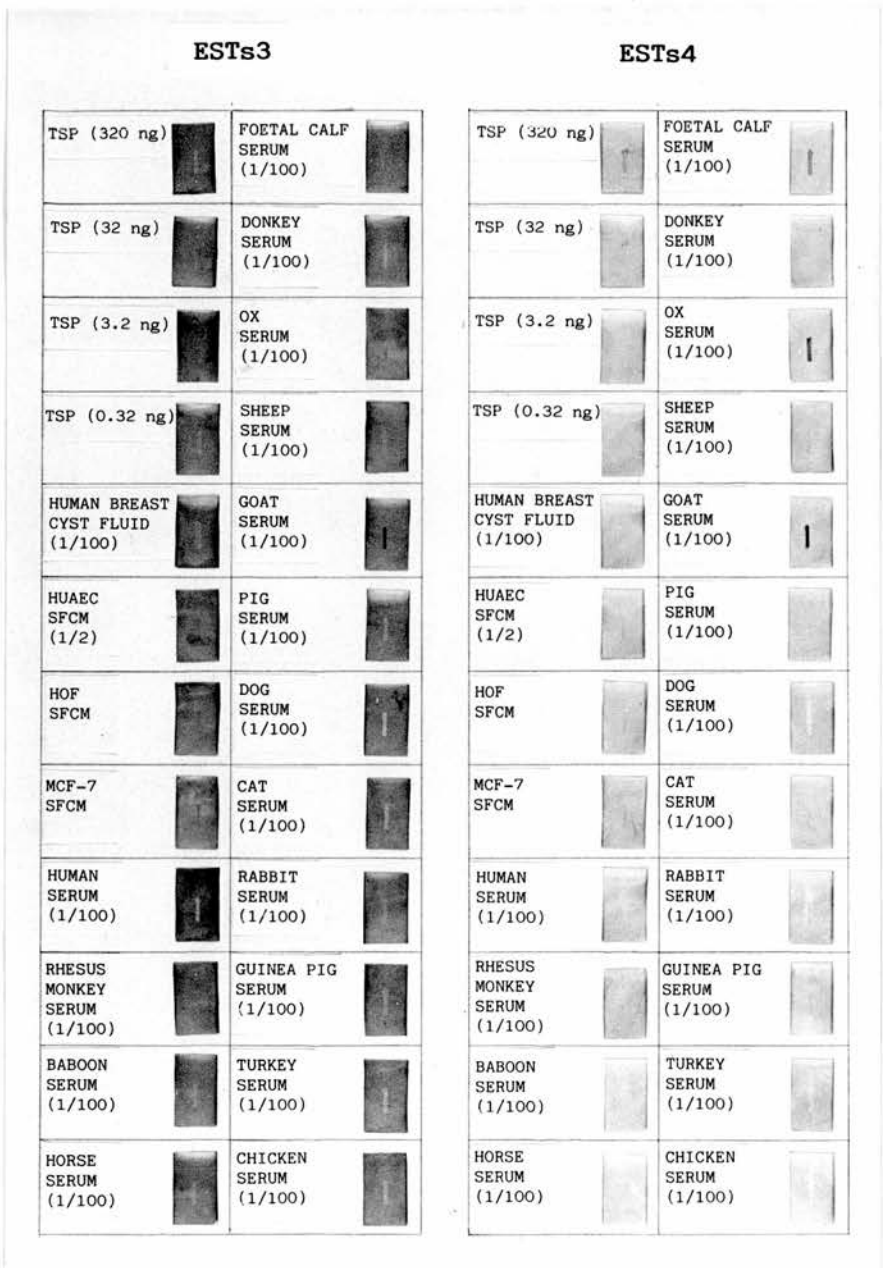
Only 4 of the 15 antibodies tested recognised a 1/100 dilution of a human breast cyst fluid which contained 48 ng TSP/100ul as measured by RIA using a rabbit polyclonal antibody (section 2.3). These antibodies (MA-I, ESTs6, F189/53.2 and F189/76.2) also gave the strongest binding to platelet TSP (positive with 0.32 ng TSP/slot) and to serum-free conditioned medium from HUAEC, HOF and MCF-7 cells.

Of the antibodies which showed appreciable binding to platelet TSP, only ESTs6 cross-reacted with all the animal sera tested. This was the only antibody which recognised turkey and chicken sera; the other animal sera were recognised variably by the remaining antibodies. An exception to this was goat serum which strongly

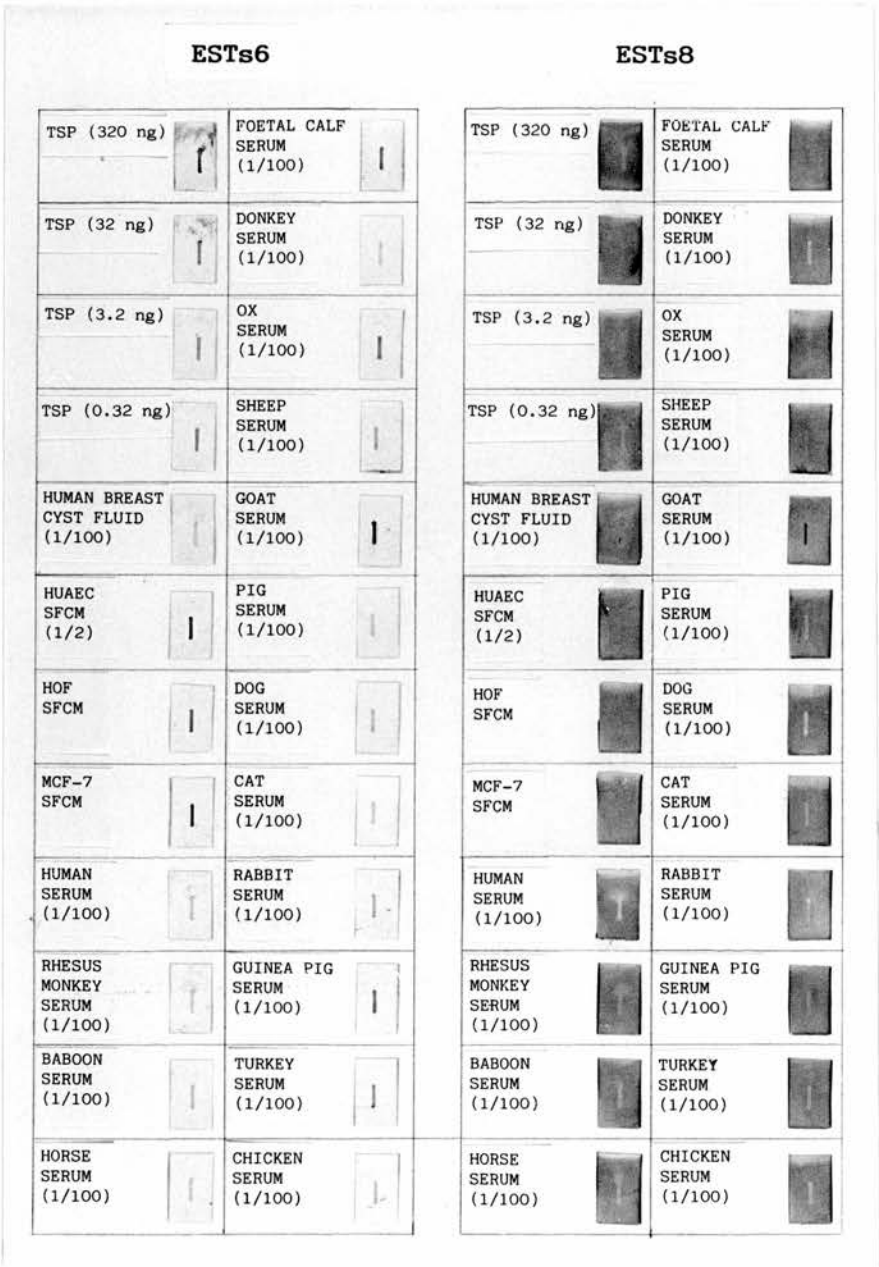
**Fig. 8-5a. Binding patterns of monoclonal anti-TSP antibodies (MA-I, MA-II) to various antigens in a SIBA**



**Fig. 8-5b. Binding patterns of monoclonal anti-TSP antibodies (ESTs3, ESTs4) to various antigens in a SIBA**



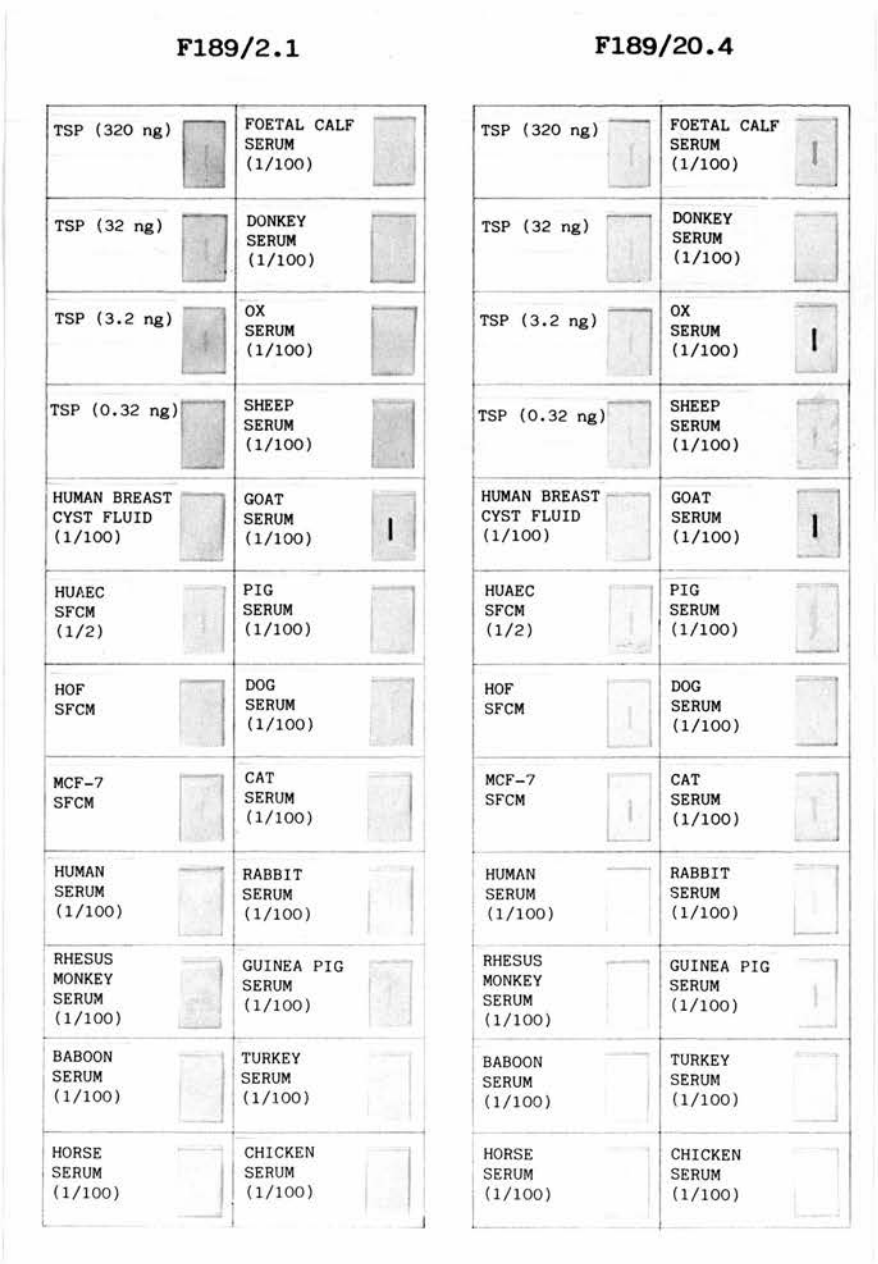
**Fig. 8-5c. Binding patterns of monoclonal anti-TSP antibodies (ESTs6, ESTs8) to various antigens in a SIBA**



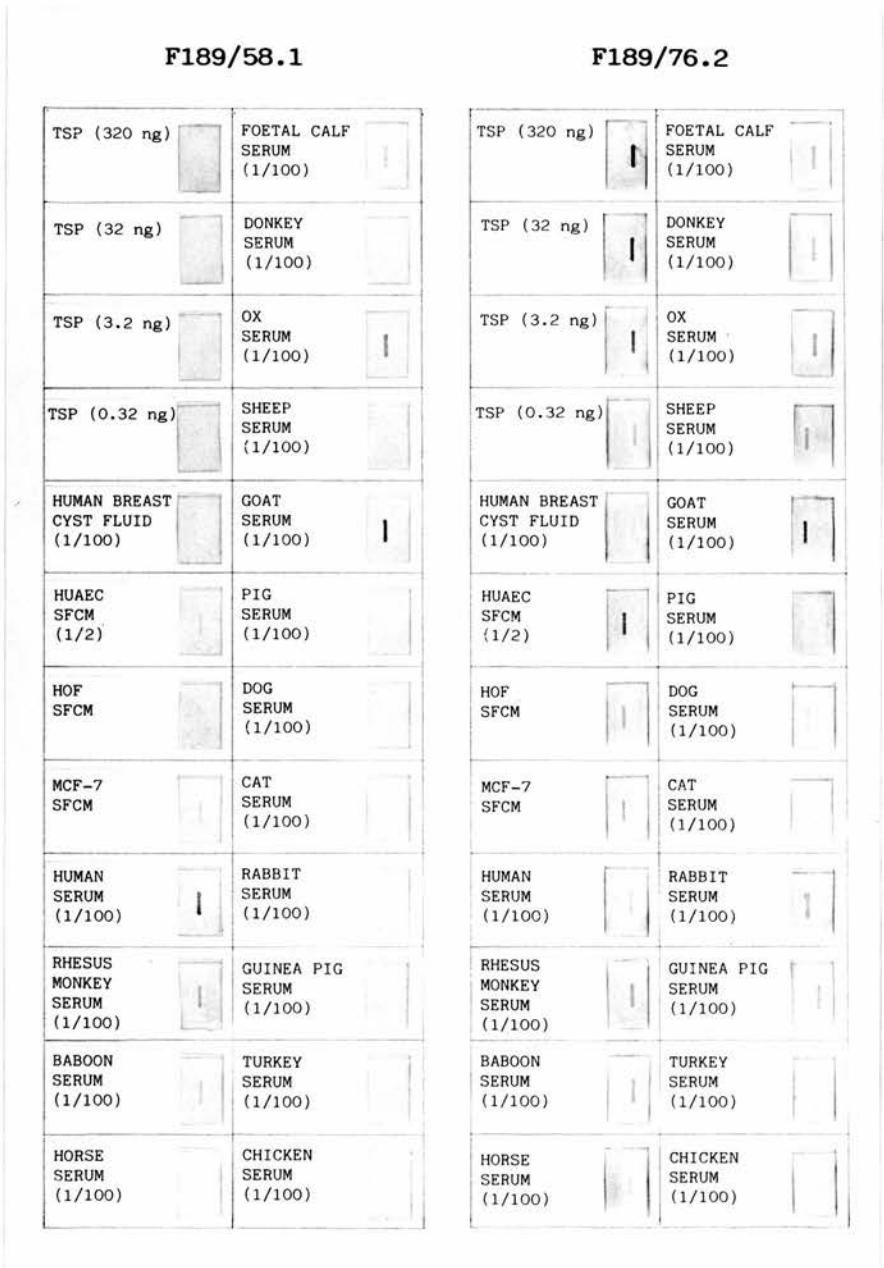
**Fig. 8-5d. Binding patterns of monoclonal anti-TSP antibodies (F189/12.3, F189/53.2) to various antigens in a SIBA**

F189/12.3		F189/53.2	
TSP (320 ng)	FOETAL CALF SERUM (1/100)	TSP (320 ng)	FOETAL CALF SERUM (1/100)
TSP (32 ng)	DONKEY SERUM (1/100)	TSP (32 ng)	DONKEY SERUM (1/100)
TSP (3.2 ng)	OX SERUM (1/100)	TSP (3.2 ng)	OX SERUM (1/100)
TSP (0.32 ng)	SHEEP SERUM (1/100)	TSP (0.32 ng)	SHEEP SERUM (1/100)
HUMAN BREAST CYST FLUID (1/100)	GOAT SERUM (1/100)	HUMAN BREAST CYST FLUID (1/100)	GOAT SERUM (1/100)
HUAEC SFCM (1/2)	PIG SERUM (1/100)	HUAEC SFCM (1/2)	PIG SERUM (1/100)
HOF SFCM	DOG SERUM (1/100)	HOF SFCM	DOG SERUM (1/100)
MCF-7 SFCM	CAT SERUM (1/100)	MCF-7 SFCM	CAT SERUM (1/100)
HUMAN SERUM (1/100)	RABBIT SERUM (1/100)	HUMAN SERUM (1/100)	RABBIT SERUM (1/100)
RHESUS MONKEY SERUM (1/100)	GUINEA PIG SERUM (1/100)	RHESUS MONKEY SERUM (1/100)	GUINEA PIG SERUM (1/100)
BABOON SERUM (1/100)	TURKEY SERUM (1/100)	BABOON SERUM (1/100)	TURKEY SERUM (1/100)
HORSE SERUM (1/100)	CHICKEN SERUM (1/100)	HORSE SERUM (1/100)	CHICKEN SERUM (1/100)

**Fig. 8-5e. Binding patterns of monoclonal anti-TSP antibodies (F189/2.1, F189/20.4) to various antigens in a SIBA**



**Fig. 8-5f. Binding patterns of monoclonal anti-TSP antibodies (F189/58.1, F189/76.2) to various antigens in a SIBA**





**Fig. 8-5g. Binding patterns of monoclonal anti-TSP antibodies (FTI 10, FTI 52, FTI 53) to various antigens in a SIBA**

FTI 10		FTI 52		FTI 53	
TSP (320 ng)	FOETAL CALF SERUM (1/100)	TSP (320 ng)	FOETAL CALF SERUM (1/100)	TSP (320 ng)	FOETAL CALF SERUM (1/100)
TSP (32 ng)	DONKEY SERUM (1/100)	TSP (32 ng)	DONKEY SERUM (1/100)	TSP (32 ng)	DONKEY SERUM (1/100)
TSP (3.2 ng)	OX SERUM (1/100)	TSP (3.2 ng)	OX SERUM (1/100)	TSP (3.2 ng)	OX SERUM (1/100)
TSP (0.32 ng)	SHEEP SERUM (1/100)	TSP (0.32 ng)	SHEEP SERUM (1/100)	TSP (0.32 ng)	SHEEP SERUM (1/100)
HUMAN BREAST CYST FLUID (1/100)	GOAT SERUM (1/100)	HUMAN BREAST CYST FLUID (1/100)	GOAT SERUM (1/100)	HUMAN BREAST CYST FLUID (1/100)	GOAT SERUM (1/100)
HUAEC SFCM (1/2)	PIG SERUM (1/100)	HUAEC SFCM (1/2)	PIG SERUM (1/100)	HUAEC SFCM (1/2)	PIG SERUM (1/100)
HOF SFCM	DOG SERUM (1/100)	HOF SFCM	DOG SERUM (1/100)	HOF SFCM	DOG SERUM (1/100)
MCF-7 SFCM	CAT SERUM (1/100)	MCF-7 SFCM	CAT SERUM (1/100)	MCF-7 SFCM	CAT SERUM (1/100)
HUMAN SERUM (1/100)	RABBIT SERUM (1/100)	HUMAN SERUM (1/100)	RABBIT SERUM (1/100)	HUMAN SERUM (1/100)	RABBIT SERUM (1/100)
RHESUS MONKEY SERUM (1/100)	GUINEA PIG SERUM (1/100)	RHESUS MONKEY SERUM (1/100)	GUINEA PIG SERUM (1/100)	RHESUS MONKEY SERUM (1/100)	GUINEA PIG SERUM (1/100)
BABOON SERUM (1/100)	TURKEY SERUM (1/100)	BABOON SERUM (1/100)	TURKEY SERUM (1/100)	BABOON SERUM (1/100)	TURKEY SERUM (1/100)
HORSE SERUM (1/100)	CHICKEN SERUM (1/100)	HORSE SERUM (1/100)	CHICKEN SERUM (1/100)	HORSE SERUM (1/100)	CHICKEN SERUM (1/100)

bound all the antibodies tested, including those which did not bind to purified human platelet TSP.

Antibodies which bound to TSP at a loading of 32 ng/slot or less also bound to HUAEC SFCM (which contained 70 ng TSP/slot) whereas MCF-7 SFCM (approximately 1 ng TSP/slot) was only recognised by antibodies with the highest affinities for TSP (positive at a loading of 3.2 ng/slot or less). An exception to this was antibody F189/58.1 which did not bind appreciably to purified human platelet TSP but recognised both HUAEC and MCF-7 SFCM. Although HOF SFCM contained a comparable amount of TSP (measured by RIA using a polyclonal antibody) as did that from HUAEC, it was not recognised by four of the antibodies which bound to the latter (MA-II, F189/12.3, F189/58.1 and FTI 10).

#### 8.7 Discussion

It was interesting that native TSP, purified in the presence of  $\text{Ca}^{2+}$ , did not appear to be immunogenic in mice whereas that prepared in the absence of  $\text{Ca}^{2+}$  produced a detectable antibody response in over half the animals tested. TSP has been described as a nodular rod-shaped molecule with certain regions stabilised by  $\text{Ca}^{2+}$  (section 1.5). Removal of  $\text{Ca}^{2+}$  may cause these parts of the polypeptide chains to unravel, producing a more filamentous structure. The greater immunogenicity of  $\text{Ca}^{2+}$ -deplete TSP may therefore be due to such a physical change, allowing some epitopes (which would not be accessible in the more tightly folded  $\text{Ca}^{2+}$ -replete form) to be presented to the host's immune system.

In order to screen for anti-TSP antibodies in the culture medium of hybridoma cells, it was necessary to develop a solid-phase

binding assay since the presence of foetal calf serum interfered with the binding of  $^{125}\text{I}$ -TSP in the modified RIA. The ELISA technique has several advantages over RIA which may allow for the more sensitive detection of antigen-antibody binding:

- 1) Adsorption of antigen onto solid phase may create a high local concentration where antibody can bind favourably despite the presence of free antigen in solution.

- 2) Solid-phase antigen is not chemically modified (unlike the iodinated TSP used in RIA) and may fully retain its immunological characteristics.

- 3) Use of an enzyme-labelled secondary antibody greatly amplifies the binding signal.

Despite the advantages of the screening ELISA, the presence of foetal calf serum could still attenuate the binding of anti-TSP antibodies which cross-react with bovine TSP (results not shown).

The high proportion of IgM antibodies which were evoked by TSP (8 out of 10 of the final MAb panel) was an unusual immune response. This finding was unlikely to have been due to the selection procedure since a standard immunisation schedule was used and the screening ELISA was at least as sensitive in the detection of IgG as IgM. Indeed, the two IgG<sub>1</sub> MAbs which were obtained had the highest titres of all the antibodies, a finding which was consistent with the general observation that IgG antibodies usually bind antigens with higher affinities than those of the IgM class. The preponderance of IgM antibodies might suggest that the immune response to TSP occurred without the involvement of helper T-cells, since T-independent antigens evoke primarily IgM antibodies (Roitt et al 1985). Furthermore, the few known T-independent antigens are

all large polymeric molecules with repeating antigenic determinants, and TSP is a trimer of identical subunits which has a tendency to self-aggregate into large molecular weight oligomers (Booth et al 1985, Clezardin et al 1986b).

Results from the CelliGen cell culture system suggested that continuous stirred culture was a suitable alternative to flat-bed culture for the bulk production of MAb. It was evident that the duration of culture was an important factor in determining the maximum antibody titre. This was optimal shortly after the number of viable cells reached its peak. As cells began to die, the anti-TSP activity decreased, possibly due to degradation of the relatively unstable IgM molecule by released enzymes and the increasingly acidic conditions.

Two different characteristics of IgG and IgM antibodies were utilised for their purification from bulk culture supernatants. IgG<sub>1</sub> (and other IgG subclasses) binds specifically to protein A, a 42 kdal polypeptide of the cell wall of *Staphylococcus aureus* (Harlow & Lane 1988). Using an immobilised form of protein A, murine anti-TSP MAbs of this subclass were separated from other constituents of the culture supernatant by affinity chromatography. Thus, IgG<sub>1</sub> molecules were specifically adsorbed onto a protein A-Sepharose 4B column at pH 8 and then desorbed by reducing the pH after non-adsorbed proteins had been washed through.

IgM antibodies do not bind to protein A but, because of their large size (molecular weight approximately 900,000), could be separated from most other proteins by gel filtration chromatography using a Sephacryl S-300 column. The IgM molecules were excluded by the Sephacryl beads and eluted close to the void volume, while

smaller proteins were retained for longer on this column.

All the anti-TSP MAbs, except ESTs3 and ESTs8, were able to recognise purified human platelet TSP adsorbed either onto polystyrene (ELISA) or onto a polyvinylidene difluoride membrane (SIBA). The well-characterised antibodies MA-I and MA-II showed unique binding patterns in the ELISA and SIBA, as did the other MAbs tested. MA-I has been shown to bind to a portion of the connecting region of TSP near the C-terminal globular domain while the epitope of MA-II is located in the N-terminal globular domain (Lawler et al 1985). It was interesting that in the ELISA, MA-II bound only to TSP which had a normal heparin-binding affinity and is directed against the heparin-binding domain of TSP, whereas MA-I recognised all the peaks from a thrombin-stimulated platelet releasate separated by heparin-affinity chromatography. It may be concluded that peaks A - D used in the ELISA contained TSP (as demonstrated by RIA and MA-I binding) but which had altered heparin-binding affinities, possibly due to partial degradation causing loss of the N-terminal globular domain, or to complex formation with other platelet  $\alpha$ -granule components. The unique binding patterns observed for each MAb in both assay systems may be evidence that they are directed against different epitopes of the TSP molecule, as has been shown for MA-I and MA-II. Without further studies however, the possibility cannot be excluded that at least some of the MAbs could be directed against other proteins which might have been present in small amounts in the TSP preparations used as immunogens.

The results from the SIBA are considered to be the first demonstration of binding of murine anti-TSP MAbs to TSP from

various sources using this technique. The order of their TSP-binding affinities was generally comparable to those observed by ELISA, with the exception of ESTs6, which had the highest affinity for human platelet TSP in the SIBA but one of the lowest by ELISA. This could indicate that TSP bound to polyvinylidene difluoride has a different conformation to that adsorbed onto polystyrene.

The binding patterns of the MAbs to the various antigens tested in the SIBA may be explained in part by their different affinities for platelet TSP. Thus, only those MAbs which showed the highest affinities for TSP also recognised a human breast cyst fluid and serum-free conditioned media from three different cell types. Although it would be interesting to speculate more, further conclusions cannot be drawn from the data without full characterisation of the MAbs.

MAbs, by nature of their single-epitope specificity, are potentially more useful than polyclonal antisera in studies of the structure and function of TSP. Given that separate domains of the TSP molecule appear to be responsible for its various activities (sections 1.3 and 1.4), it should be possible to produce MAbs against specific domains which block the functions of TSP mediated by those domains. Indeed, several such MAbs have already been described (Galvin et al 1985, Dixit et al 1985, Roberts et al 1987) and it remains to be seen whether the MAbs described here will be useful in defining further the functional organisation of the TSP molecule.

## CHAPTER NINE

### IMMUNOCYTOCHEMICAL LOCALISATION OF THROMBOSPONDIN

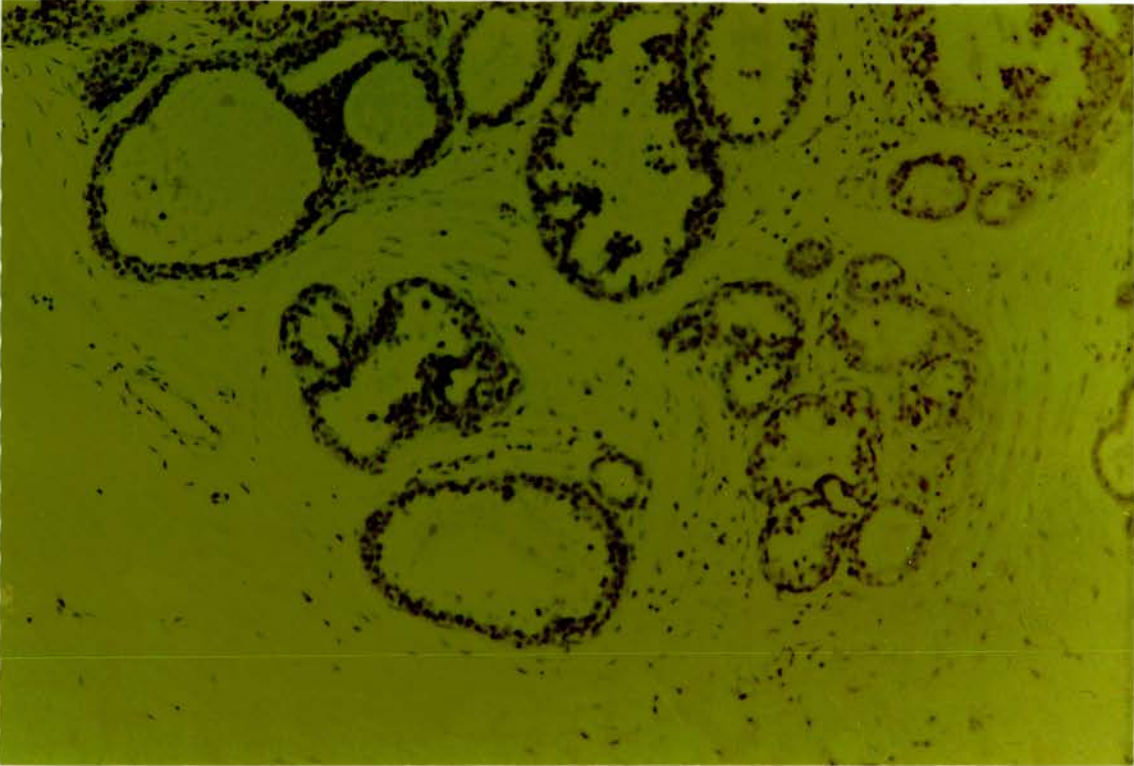
In order to investigate and define the cell types responsible for the production of TSP *in vivo*, particularly in the breast, sections of various different tissues and cytospin preparations of isolated cells were used for immunocytochemical studies with antibodies to human platelet TSP.

Formalin fixed, paraffin embedded sections of human breast cancer and lactating goat mammary gland were provided by Dr D. Harrison, Department of Pathology, University of Edinburgh. Cytospin preparations of human skin mast cells were provided by Dr M. Church, Clinical Pharmacology Group, University of Southampton. Studies of tonsil, umbilical cord and HUVEC cytospin preparations were done in collaboration with Dr N.R. Hunter, Scottish National Blood Transfusion Service Headquarters Unit Laboratory, Edinburgh.

#### 9.1 Human breast cancer

TSP was localised in human breast cancer by the PAP technique (section 2.10.2) using a specific polyclonal rabbit anti-TSP antiserum (diluted 1/10). A negative control was performed by substituting normal rabbit serum (diluted 1/10) for the 1st layer antibody. Figs. 9-1 and 9-2 show, at low magnification, a group of lobules from the control and test slides respectively. The negative

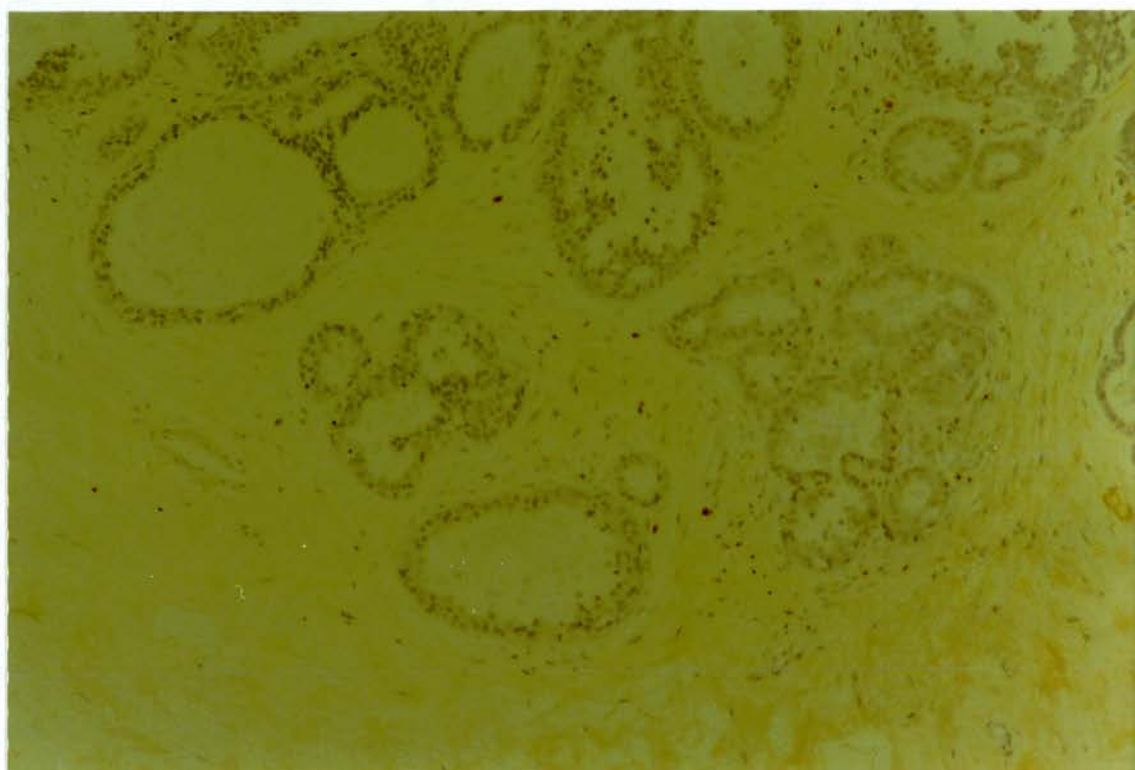
**Fig. 9-1. Human breast cancer: PAP technique, negative control**  
total magnification 170x





**Fig. 9-2. Human breast cancer: PAP technique, anti-TSP**

total magnification 170x



control had light background staining only, while the anti-TSP antiserum gave intense staining of isolated mononuclear cells scattered variably throughout the interlobular stroma, which itself was more heavily stained than in the control. Fig. 9-3 illustrates a region of the test slide at high magnification, showing intense cytoplasmic staining of one of the cells in the interlobular stroma, lighter staining of the stromal material, and absence of staining of the lobules.

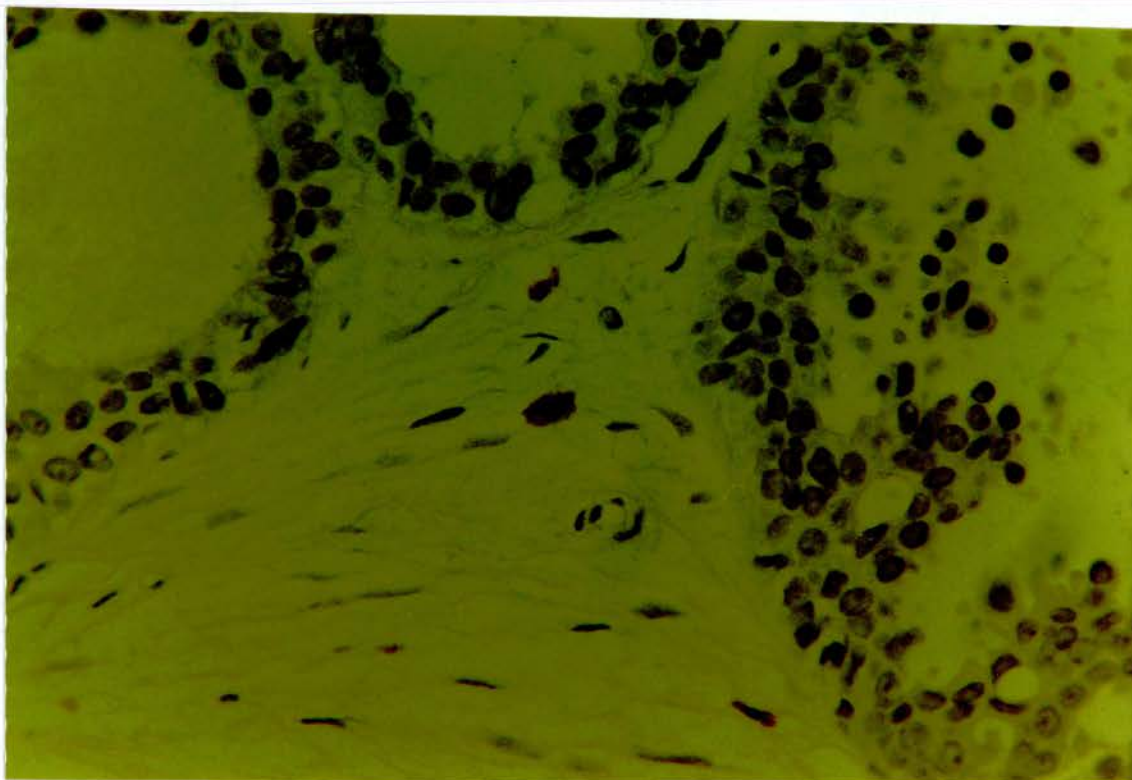
TSP was also localised in other sections of the same tissue by the indirect immunoperoxidase technique (section 2.10.1) using murine monoclonal anti-TSP antibody F189/53.2 (purified from culture supernatant, diluted 1/100). Results were similar to those obtained with the specific polyclonal antiserum, and fig. 9-4 shows, at high magnification, a group of intensely stained cells in the interlobular stroma.

Human breast cancer was also stained with toluidine blue, a basic dye which turns a characteristic purple colour upon interaction with acid mucopolysaccharides (e.g. heparin). Fig. 9-5 shows, at high magnification, three cells in the stroma which have intense cytoplasmic metachromatic staining. This distribution of staining by toluidine blue was indistinguishable from that observed during the localisation of TSP in human breast cancer by immunocytochemical techniques.

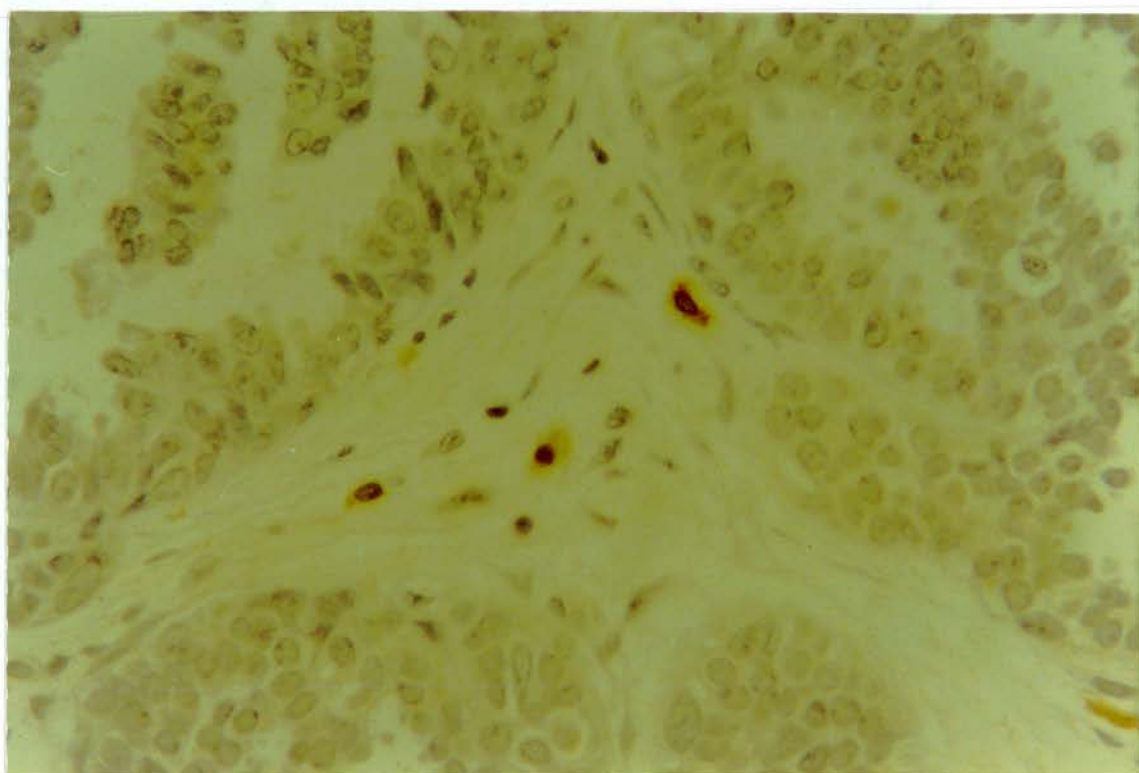
## 9.2 Goat mammary gland

Mammary gland tissue from a normal, primiparous, lactating goat was provided by Dr R. Nowak, AFRC Institute of Animal Physiology and Genetics Research, Cambridge. TSP was localised in goat mammary

**Fig. 9-3. Human breast cancer: PAP technique, anti-TSP**  
total magnification 700x

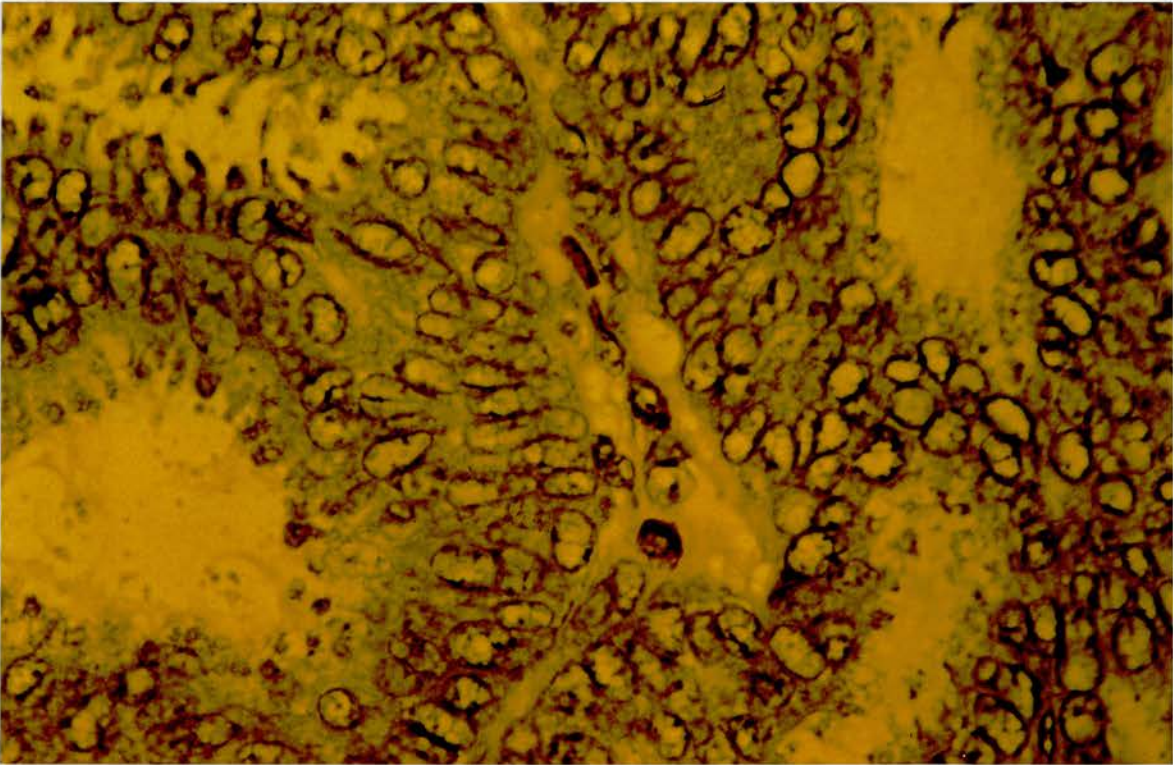


**Fig. 9-4. Human breast cancer: Indirect Immunoperoxidase, anti-TSP**  
total magnification 700x





**Fig. 9-5. Human breast cancer: Toluidine blue**



gland by the indirect immunoperoxidase technique using murine monoclonal antibody F189/53.2 (purified from culture supernatant, diluted 1/1000). A negative control was performed by substituting normal mouse serum (diluted 1/100) for the 1st layer antibody. Figs. 9-6 and 9-7 show, at low magnification, areas of mammary gland from the control and test slides respectively. The negative control had light background staining of the interlobular stroma only, while antibody F189/53.2 gave strong staining throughout the interlobular stroma and absence of staining of the lobules. Fig. 9-8 illustrates a region of the test slide at high magnification, showing strong staining of the stromal material and an absence of stained cells, either in the stroma or the lobules.

### 9.3 Other tissues and cells

Murine monoclonal anti-TSP antibodies were used to localise TSP in other human tissues and cells (results not shown).

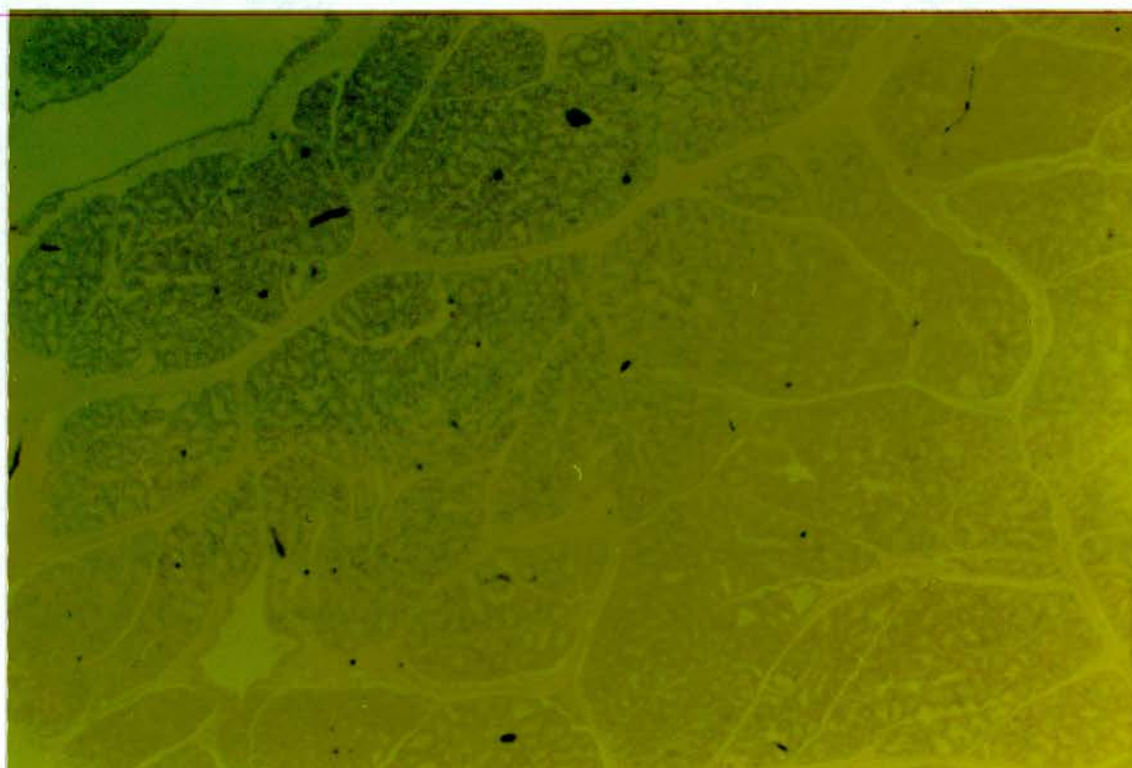
In tonsil, there was strong staining of venous and arterial endothelium, of cellular elements in the germinal centres (possibly macrophages), variable staining of connective tissue and possible staining of T-lymphocytes.

In umbilical cord, TSP was localised in venous and arterial endothelium.

Cytospin preparations of HUVEC showed granular staining of the cytoplasm, while those of mast cells isolated from human foreskin were negative.

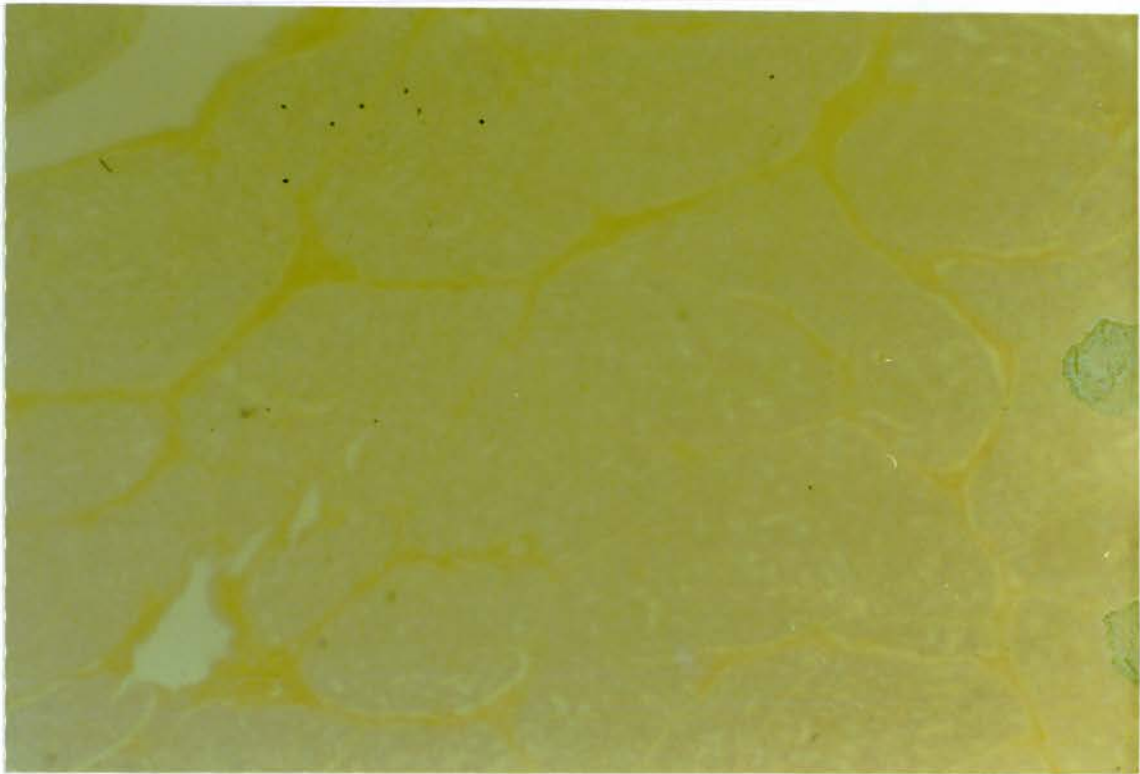
**Fig. 9-6. Goat mammary gland: Indirect Immunoperoxidase,  
negative control**

total magnification 30x



**Fig. 9-7. Goat mammary gland: Indirect Immunoperoxidase, anti-TSP**

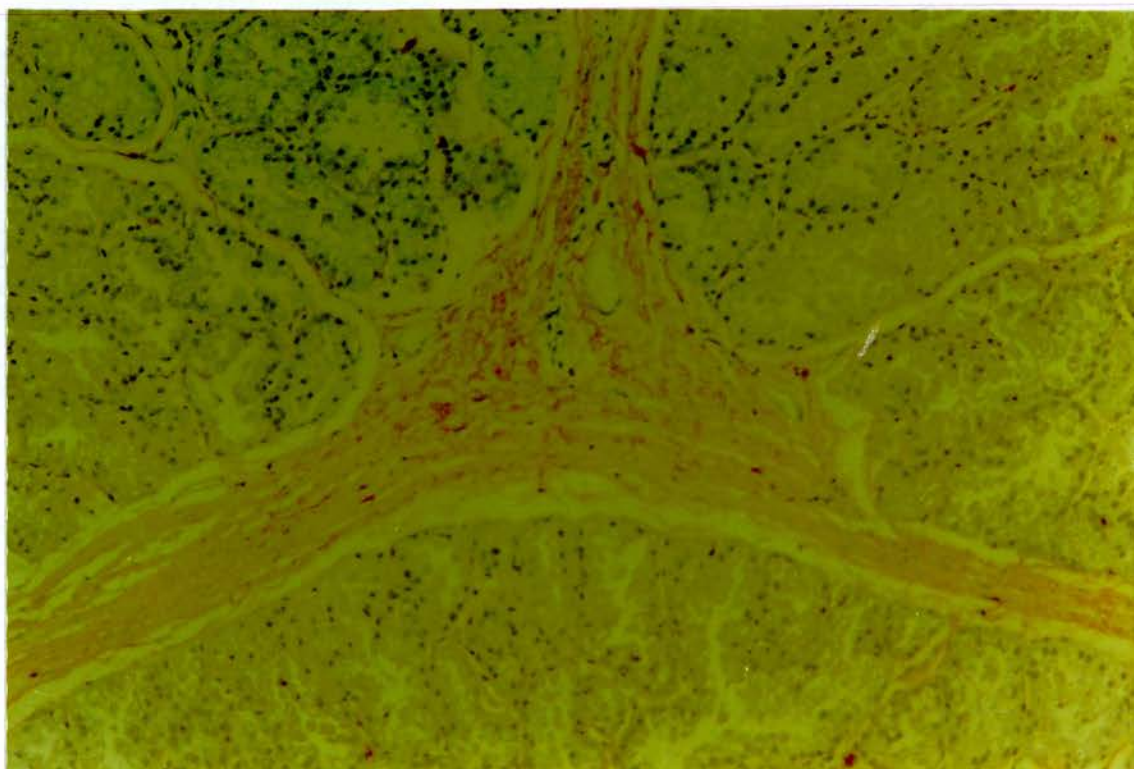
total magnification 30x





**Fig. 9-8. Goat mammary gland: Indirect Immunoperoxidase, anti-TSP**

total magnification 170x



#### 9.4 Discussion

It is surprising that, given its synthesis and secretion by a wide variety of cultured cells (section 1.5), TSP does not appear to be prevalent in tissues *in vivo*. Wight et al (1985) used immunofluorescence techniques to demonstrate the presence of TSP in human peritubular connective tissue, basement membranes at dermal-epidermal junctions and beneath glandular epithelium in skin and lung, skeletal muscle interstitial connective tissue and in small blood vessels and aorta. They concluded that TSP was a component of the extracellular matrix of some tissues while confined to basement membrane regions of others, and suggested that TSP may be a general constituent of loose connective tissues. In contrast, Murphy-Ullrich & Mosher (1987) were unable to obtain specific staining of various human adult tissues using antibodies to platelet TSP. However, they did observe extensive staining of loose connective tissue in human foetal limb, indicating that TSP may be a major component of extracellular matrix only during certain periods of development or a cell's life span. Alternatively, TSP could be present in adult tissues but not detected because of a lack in assay sensitivity, the masking of an epitope, or conformational differences which cause the antigen to be unrecognisable by the antibodies used. Indeed, evidence for such a polymorphism between platelet, endothelial cell and fibroblast TSPs has been reported (Clezardin et al 1986b).

In the present study, a restricted distribution of TSP was also observed in several different tissues and cells. Immunoperoxidase staining of tonsil and umbilical cord using specific polyclonal or monoclonal antibodies to human platelet TSP showed the presence of

TSP in connective tissue and in blood vessel endothelium, similar to the results obtained by Wight et al (1985) in other tissues. Localisation of TSP within the endothelium was supported by positive staining of cytopsin preparations of cultured HUVEC.

TSP was also strongly immunolocalised throughout the stromal connective tissue of normal lactating goat mammary gland, but only weakly in the stromal material of human breast cancer. Comparisons of non-lactating/lactating and of normal/malignant breast were not possible due to the unavailability of non-diseased breast tissue. Nevertheless, the abundance of TSP in the stroma of lactating goat mammary gland is interesting considering the high levels of TSP which have been found in goat and human milk, particularly during the initiation of lactation (Dawes et al 1987, section 5.2). The lack of staining of lobules in both normal and malignant mammary gland indicates that mammary epithelial cells are probably not a major source of TSP in these tissues, and is consistent with the low production of TSP by cultured breast cancer cells which has been observed (section 7.3).

The stroma of human breast cancer, but not that of normal goat mammary gland, contained variable numbers of intensely staining mononuclear cells. A similar distribution of stained cells was obtained using toluidine blue, which reacts with heparin (and other acid mucopolysaccharides) in mast cell granules. However, cytopsin preparations of human skin mast cells were negative for TSP, suggesting that the intensely staining cells of breast cancer stroma may be a different type of infiltrating cell. Studies of tonsil showed that TSP could be localised in cells (possibly macrophages) in the germinal centres. Also, TSP is known to be

synthesised and secreted by macrophages and monocytes (Jaffe et al 1985). Furthermore, solid human tumours (such as breast cancer) are sometimes characterised by a marked mononuclear cell infiltrate, frequently consisting of macrophages and various types of lymphocyte as well as minority populations of plasma cells and mast cells (Roitt et al 1985). This infiltration may vary from florid to none at all, and rarely follows a consistent or predictable pattern. Thus, if such cells are a major source of TSP in breast cancer, then the large variation in extent of infiltration may account for the wide range of TSP levels which have been found in malignant breast tissue (section 6.1). The question would then arise as to the possible role of TSP in macrophage function and the immune response to tumours.

## CHAPTER TEN

### GENERAL DISCUSSION

When this project was commenced in 1985, the physiological role of TSP was ill-defined, and though its involvement in platelet aggregation is well recognised, many questions regarding the function of TSP remain unanswered. By studying the production and distribution of TSP in a broad range of cells, tissues and body fluids it was hoped that some clues would emerge as to the wider importance of this glycoprotein.

The studies described in this thesis were dependent to a large extent on a regular supply of freshly prepared human platelet concentrates as the source from which TSP could be purified for use in various immunoassays, in structural studies, and as an immunogen for the generation of MAbs. No commercial TSP preparation was, nor at the present time is, available as an alternative. Since TSP is relatively labile towards proteolysis, and the yield from purification procedures is low, the availability of a suitable source of TSP may become a limiting factor in research studies.

When platelet function is normal, stimulation of these cells by thrombogenic agents and the subsequent release of  $\alpha$ -granule constituents is the major factor which determines the level of TSP in the circulation. However, extra-platelet sources such as endothelial cells and white blood cells may contribute substantially to basal plasma TSP levels as has been demonstrated

in thrombocytopenic patients, and it is not known to what extent these sources may be stimulated in various pathophysiological situations. For example vWF, an endothelial cell adhesive protein, was elevated in the plasma of patients following general surgery, possibly due to increased endothelial cell activity during wound healing, while TSP has been localised to vessel walls and is particularly abundant in atherosclerotic lesions (Wight et al 1985).

The long half-life of TSP in the circulation is probably related to its high molecular weight (making TSP too large to be filtered at the glomerulus). TSP catabolism most likely occurs in the liver, but little is known of this excretory pathway; further studies to define the mechanisms involved would be helpful in understanding the control of circulating TSP levels and the role of TSP in haemostasis and thrombosis.

Measurement of plasma levels of  $\alpha$ -granule proteins is not a reliable indicator of the extent of in vivo platelet activation in clinical situations where rates of catabolism and clearance are modulated by disease. The potential use of surface-associated TSP measurements on fixed, isolated platelets as an alternative to plasma concentrations for assessing platelet activation is worthy of further study and may find clinical use in many conditions including thrombosis, vascular disease, myeloproliferative disorders, leukaemia and diabetes.

Novel studies of TSP in the breast have increased the breadth of knowledge regarding its occurrence and significance in biological fluids and tissues. The remarkably high levels of TSP in type II cyst fluids, early milk, other breast secretions and malignant

breast tissue suggest that TSP may have an important function in the mammary gland, although no physiological role has yet been identified. However, the data may point to an involvement of TSP in the activity of certain white blood cells (possibly macrophages) which infiltrate the interstitial connective tissues of the breast, particularly during the peripartum period and in breast cancer. It may be that TSP has a role in macrophage function which is of equal physiological importance as that which has long been recognised in platelets. Further work aimed at defining such a role for TSP and its possible involvement in the interactions of macrophages, the immune system and malignant cells may prove to be most interesting.

Cultured cell systems, similar to those described in chapter 8, will continue to be useful models for studying the synthesis and secretion of TSP and its role in cell-cell and cell-matrix interactions. The increasing availability of MAbs which can inhibit specific functions of TSP will allow the molecular mechanisms underlying such interactions to be investigated.

To date, little is known of the functional importance of the carbohydrate moiety of TSP. Although the full amino acid sequence of TSP has now been determined, knowledge of the structural organisation of its sugar residues remains incomplete. The possibility exists for variation in the carbohydrate moiety between TSPs produced by different cell types, which could give rise to altered functional properties. Research along these lines may lead to a greater understanding of how TSP could be involved in such apparently diverse physiological processes as platelet aggregation, fibrinolysis, cell-matrix interactions, tissue development, wound healing, immune responses and malignant disease.

In summary, the results presented in this thesis support current knowledge of TSP as an adhesive glycoprotein of platelets, vessel walls and connective tissues, but indicate that TSP is more ubiquitous than previously thought. While platelets are the major source of TSP in the circulation, different cell types appear to be responsible for the production of this glycoprotein in other body fluids and in tissues. Novel studies in the breast have revealed a marked association of TSP with cancerous tissue and some cyst fluids which could be due to production by vascular endothelium and certain white blood cells. While the role of TSP in the breast remains to be elucidated, its relation to disease states may be of particular physiological importance.



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## APPENDIX

### PUBLISHED PAPERS

Clezardin P, Hunter NR, Lawler JW, Pratt DA, McGregor JL, Pepper DS, Dawes J (1986). Structural and immunological comparison of human thrombospondins isolated from platelets and from culture supernatants of endothelial cells and fibroblasts: evidence for a thrombospondin polymorphism. *Eur J Biochem* **159**, 569-579.

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## Structural and immunological comparison of human thrombospondins isolated from platelets and from culture supernatants of endothelial cells and fibroblasts

### Evidence for a thrombospondin polymorphism

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Thrombospondin is a 450-kDa glycoprotein secreted by a variety of cells including endothelial cells, fibroblasts and platelets. The aim of this study was to compare the structural and immunological properties of human endothelial, fibroblast and platelet thrombospondins. All three thrombospondins were purified, digested with thermolysin, and the subsequent thermolysin-generated fragments isolated on a Superose 12 gel-permeation column using non-denaturing conditions. Each isolated proteolytic fragment of thrombospondins was then detected using either a radioimmunoassay with a polyclonal antibody or an enzyme-linked immunosorbent assay with three monoclonal antibodies (P10, MA-I, MA-II) directed against different epitopes of whole platelet thrombospondin. The fragmentation pattern of human endothelial thrombospondin consists of six major thermolysin-generated fragments (135–110, 98–82, 54–47, 25–20, 18–15 and 10 kDa) having molecular masses very similar to those observed with human fibroblast thrombospondin (115–100, 92–80, 54–49, 27–21, 17–13 and 12–10 kDa). Treatment of platelet thrombospondin with thermolysin only generated four proteolytic fragments having molecular masses of 110, 50, 25 and 12/10 kDa respectively. All these proteolytic fragments of endothelial, fibroblast and platelet thrombospondins were recognized by a polyclonal antibody. Monoclonal antibodies MA-I and P10 essentially recognized two proteolytic fragments (135–110, 98–82 kDa) of endothelial and fibroblast (115–100, 92–80 kDa) thrombospondins, and the 110-kDa fragment of platelet thrombospondin. Monoclonal antibody MA-II recognized three proteolytic fragments (54–47, 25–20, 18–15 kDa) of endothelial and fibroblast (54–49, 27–21, 17–13 kDa) thrombospondins, and two fragments (50, 25 kDa) of platelet thrombospondin, different from those detected by P10 and MA-I. The results clearly demonstrate that, under non-denaturing conditions, endothelial and fibroblast thrombospondins are structurally different from platelet thrombospondin since two fragments of endothelial thrombospondin (98–82, 18–15 kDa), equivalent to those of fibroblast thrombospondin (92–80, 17–13 kDa), are not released from platelet thrombospondin after thermolysin treatment. These three forms of thrombospondin are, however, immunologically indistinguishable. To investigate further the structural differences observed between platelet and the two other forms of thrombospondin, their degree of polymerization was compared. Prior to thermolysin treatment, the three forms of thrombospondin were separated into several oligomers ranging from 450 kDa to 3300 kDa when injected onto a Superose 6 gel-permeation column. In the presence of thermolysin, platelet thrombospondin oligomers were all digested whereas the 3300 kDa oligomer of endothelial or fibroblast thrombospondin was unaffected. All these oligomers were recognized by a polyclonal antibody and three monoclonal antibodies directed against platelet thrombospondin. Such a resistance to proteolysis of endothelial and fibroblast thrombospondins does explain the structural differences observed in this study, and therefore suggests that the conformational properties of these two forms of thrombospondin are different from those of platelet thrombospondin.

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**Abbreviations.** HUVEC, Human umbilical cord vein endothelial cells; HUAEC, human umbilical cord artery endothelial cells; HSVEC, human saphenous vein endothelial cells; HOF, human oral fibroblasts; HFF, human foreskin fibroblasts; HSF, human forearm skin fibroblasts; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; TMB, 3,3',5,5'-tetramethylbenzidine.

**Enzymes.** Thermolysin (EC 3.4.24.4); horseradish peroxidase (EC 1.11.1.7).

Thrombospondin is secreted from human blood platelet  $\alpha$ -granules following stimulation [1] and binds to the surface of activated platelets in the presence of physiological concentrations of calcium [2]. This molecule is also secreted and incorporated into the extracellular matrix by a variety of cultured cells including endothelial cells [3, 4], fibroblasts [5, 6], smooth muscle cells [5], monocytes [7] and some macrophages [7, 8].

Thrombospondin, in its native state, is a glycoprotein composed of four globular regions stabilized with calcium,



which are unravelled in the presence of EDTA [9]. This unravelling results in an increased susceptibility of thrombospondin to protease attack [9]. Thrombospondin has a molecular mass of 450 kDa [9] and forms oligomers ranging from a dimer to a 40000-kDa polymer when released from stimulated platelets [10]. The native monomeric form of thrombospondin is composed of three equivalent disulphide-linked chains of 50–160 kDa [9]. Each thrombospondin chain is made up of several protease-resistant domains, which differ in their biological activities [11–19]. Some of these functional domains have been separated by limited proteolysis and subsequent affinity chromatography using different ligands [12, 9–22].

Following isolation of trypsin-generated and thermolysin-generated fragments of thrombospondin by gel-permeation chromatography (FPLC), and immunodetection of each isolated proteolytic fragment by a panel of polyclonal [23] and monoclonal antibodies [24] specific for platelet thrombospondin, we recently reported a polymorphism between human thrombospondins purified from platelets and foetal vein endothelial cells cultivated at confluence [25]. However, since changes in the glycosylation of glycoproteins take place in human endothelial cells as they approach confluence in culture [26], we could not exclude from our preliminary study [25] the possibility that such changes occurred in the carbohydrate moiety of endothelial thrombospondin when purified from confluent cells, thus accounting for the structural differences previously observed between the two forms of thrombospondin. In addition, qualitative and quantitative differences occur in the secretory phenotypes of foetal and adult endothelial cells [27–29]. For these reasons, we are reporting in this study the comparison of the structural and immunological properties of human thrombospondins isolated from platelets and from supernatants of foetal and adult endothelial cells cultivated at subconfluence and confluence. In addition we have extended our study to the structural and immunological characterization of human thrombospondin isolated from adult fibroblasts cultivated at confluence and subconfluence. The thermolysin fragmentation patterns of platelet, endothelial and fibroblast thrombospondins as well as their degree of polymerization were compared by gel-permeation fast liquid chromatography under non-denaturing conditions, followed by the immunological characterization of each isolated proteolytic fragment using a polyclonal [23] and three monoclonal antibodies (P10, MA-I, MA-II), two of them (P10 and MA-II) directed against functional domains of platelet thrombospondin [30, 31]. The results indicate that, whatever the foetal or adult origin of the cells may be, endothelial and fibroblast thrombospondins are structurally different from platelet thrombospondin.

## EXPERIMENTAL PROCEDURE

FPLC separations were either performed on a Superose 12 HR 10/30 or a Superose 6 HR 10/30 gel-filtration column (300 × 10 mm) (Pharmacia, Uppsala, Sweden). The Superose 12 and 6 columns separate proteins ranging from 1 kDa to 500 kDa and 5 kDa to 5000 kDa respectively. Thermolysin was from Sigma (St Louis, MO, USA). Materials and chemicals, unless stated otherwise, were as previously indicated [24, 25, 31, 32].

## Cell culture

Human foetal and adult endothelial cells from eight donors were obtained from the following sources: H7, H11, H24, H32 and H55 were foetal endothelial cells isolated from the human umbilical cord vein; H56 was foetal endothelial cells isolated from the human umbilical cord artery; LAY2 and SAP2 were adult endothelial cells isolated from the human saphenous vein. Human adult fibroblasts from four donors were obtained from the following sources: F34 was fibroblasts isolated from the normal human foreskin (a generous gift of Dr G. Priestley, Edinburgh); F5 and F22 were fibroblasts isolated from the normal human forearm skin (a generous gift of Dr G. Priestley); C1 was normal human oral fibroblasts (a generous gift of Dr Y. Barlow, Edinburgh). Human endothelial cells and fibroblasts were cultured using methods and materials previously described [29]. Cell supernatants were harvested at subconfluence and confluence.

## Purification of human thrombospondins from platelets, endothelial cells and fibroblasts

Thrombospondins secreted from thrombin-stimulated platelets and into the culture medium of human endothelial cells and fibroblasts were purified by a modification of the methods of Lawler et al. [9] and Clezardin et al. [32]. Briefly the culture medium (50–100 ml) from both endothelial cells and fibroblasts or the supernatant (15–30 ml) from thrombin-stimulated platelets were loaded onto a heparin-Sepharose column (120 × 20 mm) equilibrated in 20 mM Tris buffer, pH 7.4, containing 0.35 M NaCl (buffer A). The column was washed successively with 0.35 M, 0.55 M and 2 M NaCl in buffer A. The thrombospondin peak eluting at 0.55 M NaCl was then applied to a gelatin-Sepharose column previously equilibrated in buffer A containing 0.55 M NaCl. The flow-through material from the gelatin-Sepharose column was then concentrated (0.5–0.7 ml) and gel-filtered through a Superose 12 column connected to a FPLC system and equilibrated with 20 mM triethanolamine buffer, pH 7.4, containing 0.35 M NaCl. The thrombospondin peak (2 ml) eluted from the Superose 12 column at a molecular mass of 450 kDa. Some thrombospondin preparations were further purified on a Mono Q anion-exchange column connected to a FPLC system following a previously described method [32].

## Thermolysin digestion of purified thrombospondins

Thermolysin digestion was performed using purified thrombospondin prepared in the presence of 20 mM triethanolamine (pH 7.4) and 0.35 M NaCl. The protease to thrombospondin ratio was 1:20 (w/w) at 37°C. After 90 min of incubation the digestion of thrombospondin was terminated by addition of 10 mM EDTA. In some experiments a negative control was included by incubating purified thrombospondin with 10 mM EDTA prior to thermolysin treatment. Under such experimental conditions no proteolytic degradation of thrombospondin occurred, indicating a total inhibition of the enzymatic activity of thermolysin by EDTA as reported by Clezardin et al. [33].

## Isolation of thrombospondin proteolytic fragments by gel-permeation chromatography

The Superose 12 and 6 HR 10/30 gel-permeation columns were used to compare the thermolysin fragmentation patterns

platelet, endothelial and fibroblast thrombospondins. Prior to chromatography the Superose 12 and 6 columns were equilibrated with 20 mM triethanolamine buffer (pH 7.4) containing 0.35 M NaCl. Samples of thrombospondin digests (5–0.7 ml) were directly injected onto the column at a flow rate of 0.5 ml/min.

*Immunodetection of thrombospondin proteolytic fragments using a radioimmunoassay with a polyclonal antibody directed against platelet thrombospondin*

Using a polyclonal antibody the isolated proteolytic fragments of thrombospondins were immunodetected by competitive radioimmunoassay following a method previously described by Dawes et al. [23]. Briefly, 50 µl of each isolated proteolytic fragment were mixed with <sup>125</sup>I-labelled platelet thrombospondin (50 µl 10 ng/ml) and a rabbit polyclonal antibody (50 µl, 1:1200 dilution) directed against whole platelet thrombospondin. After incubation overnight at 4°C, 50 µl buffer containing 8.3 µl Sepharose-4B-coupled monkey anti-rabbit IgG were added, and the tubes were incubated with shaking for 45 min at 20°C. Sepharose-bound material was separated from that remaining in the liquid phase by sedimentation of the solid phase through assay buffer containing 10% (w/v) sucrose and removal of the supernatant by aspiration.

*Immunodetection of thrombospondin proteolytic fragments using an enzyme-linked immunosorbent assay with three monoclonal antibodies directed against different epitopes of whole platelet thrombospondin*

The mouse monoclonal antibodies used had been previously characterized [30, 31]. Mouse monoclonal antibodies MA-11 [30] and P10 [31] were directed against epitopes located within the heparin-binding domain and near the hemagglutinin activity domain respectively. MA-1 [30] was directed against a 47-kDa tryptic fragment of platelet thrombospondin as judged by sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis. When assayed in a radioimmunoassay it was observed that the buffer used to equilibrate the Superose 12 and 6 columns affected the binding of monoclonal antibody P10 to the isolated thrombospondin proteolytic fragments whereas the binding of the polyclonal antibody was not affected. Such an observation has also been reported by Dawes et al. [34] using different monoclonal antibodies directed against human  $\alpha$ -thrombin. The unusual behaviour of P10 explains the immunological differences previously observed between platelet and foetal endothelial thrombospondins [25]. For this reason the proteolytic fragments of thrombospondins were immunodetected by an enzyme-linked immunosorbent assay following modifications of the method described by Bos et al. [35]. Briefly, 100 µl of each isolated proteolytic fragment were added in duplicate to 96 microtitre plates with flat-bottom wells (Linbro/Titertek, Flow Laboratories Inc.) and incubated 48 h at room temperature. Negative and positive controls were phosphate-buffered saline instead of proteolytic fragments (negative control) and purified thrombospondin (positive control). After incubation, the wells were washed four times with 250 µl/well phosphate-buffered saline containing 0.1% Tween 20 (v/v) (washing buffer). The last wash was left for 30 min at room temperature. After removing the washing buffer, 100 µl anti-thrombospondin mouse IgG monoclonal antibody (diluted to

1/10000) were added to each well for 1 h incubation at 37°C. At this stage a mouse IgG monoclonal antibody not directed against thrombospondin was used at a dilution of 1/10000 as a second negative control. After incubation, the contents of the wells were aspirated and rinsed four times with the washing buffer. 100 µl goat anti-mouse IgG conjugated with horseradish peroxidase (diluted to 1/3000) (Bio-Rad Laboratories) were added to each well for an additional 1 h incubation at 37°C. The contents of the wells were then aspirated and rinsed four times with the washing buffer. 100 µl dimethylsulfoxide containing 42 mM 3,3',5,5'-tetramethylbenzidine (TMB) were mixed with 10 ml 0.1 M sodium/acetate/citric acid buffer, pH 6.0 (TMB solution). Just before use, the substrate solution was constituted by adding 100 µl 1.3 mM hydrogen peroxide to the TMB solution. Finally 100 µl/well of this substrate solution were added and the enzyme reaction was allowed to proceed for 10 min in the dark at room temperature. The reaction was then stopped with 100 µl/well 2.5 M sulphuric acid. The absorbance was read at 450 nm using a Titertek photometer (Flow Laboratories).

## RESULTS

*Isolation of thermolysin-generated fragments of thrombospondin purified from human umbilical cord vein endothelial cells (HUVEC) cultivated at subconfluence and confluence*

Thrombospondin purified from the confluent foetal vein endothelial cells (HUVEC line H11 P9) showed a single peak at 450 kDa when injected on a Superose 12 gel-permeation column (Fig. 1A). Thermolysin digestion of H11 P9 endothelial thrombospondin from confluent cells generated six major peaks, two of which eluted after the total volume of the column (Fig. 1B). The proteolytic fragments corresponding to these peaks had molecular masses (120, 95, 53, 24, 15 and 10 kDa) (Table 1) very similar to those observed after thermolysin digestion of endothelial thrombospondin purified from the same foetal cell line (H11 P9) cultivated at subconfluence (Fig. 1C and Table 1). In addition the thermolysin digestion of thrombospondin purified from four other confluent foetal vein endothelial cell lines (H24, H32, H7 and H55) gave proteolytic fragments with molecular masses very close to those previously mentioned (Table 1). Despite similarities in the molecular masses of these proteolytic fragments, their respective elution profiles from the Superose 12 column may vary considerably from one cell line to another (results not shown).

*Isolation of thermolysin-generated fragments of thrombospondin purified from human umbilical cord artery (HUAEC) and human saphenous vein endothelial cells (HSVEC) cultivated at subconfluence and confluence*

In order to ascertain whether the thermolysin fragmentation pattern of endothelial thrombospondin was unique, we have compared the structural properties of thrombospondin from foetal vein endothelial cells (HUVEC) with those of thrombospondin purified from foetal artery and adult vein endothelial cells.

The thermolysin digestion of thrombospondin purified from the confluent foetal vein endothelial cells (HUVEC line H11 P9) generated six main peaks, two of which eluted after



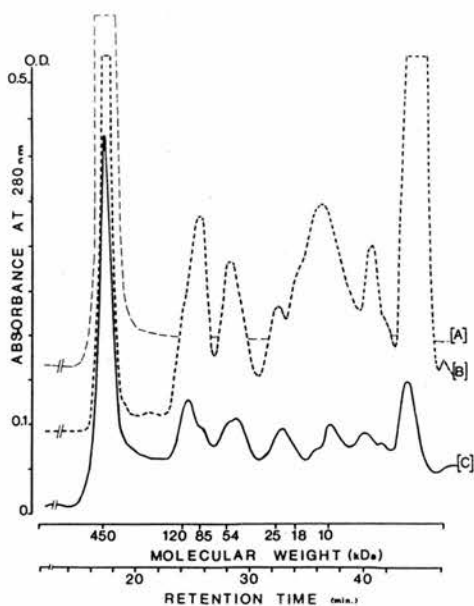


Fig. 1. Comparison of the thermolysin-generated fragments of thrombospondin purified from human umbilical cord vein endothelial cells (HUVEC) cultivated at subconfluence and confluence. (A) Thrombospondin purified from the HUVEC line H11 P9. (B) Thermolysin digests of H11 P9 endothelial thrombospondin purified from confluent cells. (C) Thermolysin digests of H11 P9 endothelial thrombospondin purified from subconfluent cells. The thermolysin/thrombospondin ratio was 1:20 (w/w). After 90 min of incubation at 7°C the thermolysin digestion of thrombospondin was terminated by addition of 10 mM EDTA. A Superose 12 gel-permeation column connected to a FPLC system was equilibrated with 20 mM triethanolamine buffer (pH 7.4) containing 0.35 M NaCl. Samples of thrombospondin digests (0.5–0.7 ml) were directly injected onto the column at a flow rate of 0.5 ml/min

the total volume of the column (Fig.2A). The proteolytic fragments corresponding to these peaks (120, 95, 53, 24, 15 and 10 kDa) (Table 1) had molecular masses very similar to those observed after thermolysin digestion of thrombospondin purified from both confluent foetal artery (HUAEC line H56) (Fig.2B and Table 1) and adult vein endothelial cells (HSVEC lines LAY2 and SAP2) (Fig.2C and Table 1). In addition, the thermolysin fragmentation patterns of thrombospondin isolated from foetal artery (HUAEC line H56 P5) and adult vein endothelial cells (HSVEC line LAY2 P5) were similar at subconfluence and confluence (Table 1).

However, when the thermolysin fragmentation patterns of foetal and adult endothelial thrombospondins (Fig.2A–C) were compared with that of platelet thrombospondin (Fig.2D), clear differences were observed. Two proteolytic fragments of 98–82 kDa and 18–15 kDa, respectively, were absent after thermolysin digestion of platelet thrombospondin (Table 1).

*Isolation of thermolysin-generated fragments of thrombospondin purified from human oral (HOF), foreskin (HFF) and skin (HSF) fibroblasts cultivated at confluence and subconfluence*

Thrombospondin purified from the confluent adult oral fibroblasts (HOF line C1) showed a single peak at 450 kDa

Table 1. Thermolysin-generated fragments of human endothelial thrombospondin obtained after gel-permeation chromatography on a Superose 12 column

In the cell denominations P followed by a number indicates the passage number. The molecular mass value of each proteolytic fragment of platelet thrombospondin represents an average obtained throughout the course of this study

Origin of cells	Denomination	Proteolytic fragments						
		kDa						
HUVEC	H24 P8	450	120	85	50	22	16	10
HUVEC	H32 P4	450	120	98	54	25	18	10
HUVEC	H7 P12	450	120	82	54	25	15	10
HUVEC	H55 P6	450	120	82	52	20	16	10
HUVEC	H11 P9 <sup>a</sup>	450	115	87	50	20	15	10
HUVEC	H11 P9	450	120	95	53	24	15	10
HUVEC	H11 P10	450	135	84	47	21	15	10
HSVEC	LAY2 P5 <sup>a</sup>	450	120	90	54	24	15	10
HSVEC	LAY2 P5	450	120	90	54	22	16	10
HSVEC	SAP2 P5	450	115	95	54	24	15	10
HSVEC	SAP2 P6	450	115	95	54	23	16	10
HUAEC	H56 P4	450	110	85	45	23	17	10
HUAEC	H56 P5 <sup>a</sup>	450	120	90	54	25	15	10
HUAEC	H56 P5	450	110	85	50	24	16	10
HUAEC	H56 P6	450	110	87	47	22	17	10
Human platelets		450	110		50	25		12/10

<sup>a</sup> Cells cultured at subconfluence.

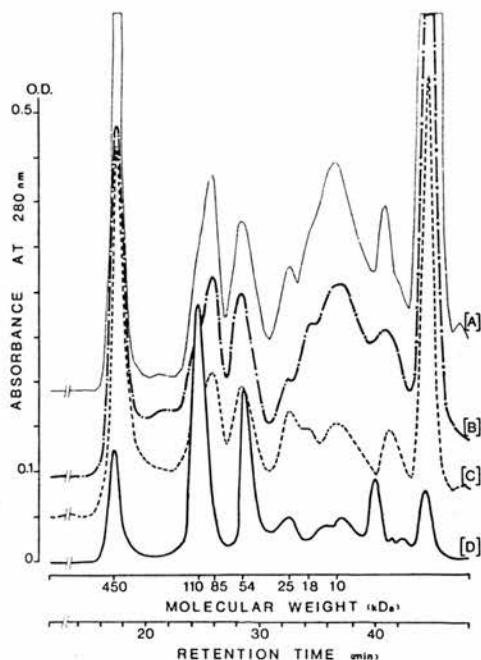


Fig. 2. Comparison of the thermolysin-generated fragments of thrombospondin purified from human foetal and adult endothelial cells with those of thrombospondin purified from human platelets. (A) Thermolysin digests of thrombospondin purified from human foetal vein endothelial cells (HUVEC line H11 P9). (B) Thermolysin digests of thrombospondin purified from human foetal artery endothelial cells (HUAEC line H56 P5). (C) Thermolysin digests of thrombospondin purified from human adult saphenous vein endothelial cells (HSVEC line SAP2 P5). (D) Thermolysin digests of thrombospondin purified from human platelets. Experimental conditions of protease digestion and chromatography were similar to Fig. 1

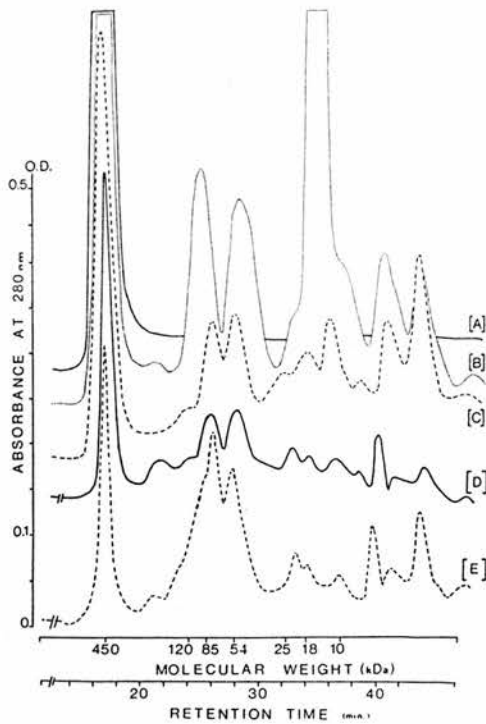


Fig. 3. Comparison of the thermolysin-generated fragments of thrombospondin purified from human oral, foreskin and forearm skin fibroblasts cultivated at confluence and subconfluence. (A) Thrombospondin purified from human oral fibroblasts (HOF line C1). (B) Thermolysin digests of thrombospondin purified from human oral fibroblasts (HOF line C1). (C) Thermolysin digests of thrombospondin purified from human foreskin fibroblasts (HFF line F34). (D) Thermolysin digests of thrombospondin purified from human forearm skin fibroblasts cultivated at confluence (HSF line F5). (E) Same as (D) cultivated at subconfluence. Experimental conditions of protease digestion and chromatography were similar to Fig. 1.

when injected on a Superose 12 gel-permeation column (Fig. 3A). Thermolysin digestion of C1 fibroblast thrombospondin generated five major peaks, two of which eluted after the total volume of the column (Fig. 3B). The proteolytic fragments corresponding to these peaks had molecular masses of 230, 100, 84, 50, 20, 12 and 10 kDa (Table 2) and were similar to those obtained after thermolysin digestion of thrombospondin purified from confluent adult foreskin fibroblasts (HFF line F34) (Fig. 3C) and adult skin fibroblasts (HSF line F5) cultivated at confluence and subconfluence respectively (Fig. 3D–E and Table 2). Similar results were obtained with thrombospondin purified from subconfluent adult oral fibroblasts (Table 2). However, the elution profile of these proteolytic fragments may vary considerably from one cell line to another (Fig. 3).

Comparison of the thermolysin-generated fragments of adult fibroblast thrombospondins with those of platelet thrombospondin, clearly showed the absence of two proteolytic fragments (92–80 kDa and 17–13 kDa) from the fragmentation pattern of platelet thrombospondin (Table 2). An additional proteolytic fragment of 230–200 kDa, present in the fragmentation pattern of fibroblast thrombospondin, was also observed after thermolysin digestion of endothelial and platelet thrombospondins when using a higher protein loading onto the Superose 12 column (results not shown).

Table 2. Thermolysin-generated fragments of human fibroblast thrombospondin obtained after gel permeation chromatography on a Superose 12 column

In the cell denominations P followed by a number indicates the passage number. The molecular mass value of each proteolytic fragment of platelet thrombospondin represents an average obtained throughout the course of this study

Origin of cells	Denomination	Proteolytic fragments	kDa									
			450	200	115	92	50	22	15	10		
HOF	C1	P4 <sup>a</sup>	450	200	115	92	50	22	15	10		
HOF	C1	P4	450	230	100	84	50	24	15	10		
HFF	F34	P5	450	200	100	80	50	27	14	12		
HFF	F34	P6	450	230	110	80	54	27	13	10		
HSF	F5	P13 <sup>a</sup>	450	220	105	85	54	24	17	10		
HSF	F5	P13	450	195	110	80	49	23	17	12		
HSF	F22	P15	450	230	115	90	50	21	17	10		
Human platelets			450		110		50	25		12/10		

<sup>a</sup> Cells cultured at subconfluence.

#### Immunological characterization of thermolysin-generated fragments of endothelial, fibroblast and platelet thrombospondins using a polyclonal antibody directed against platelet thrombospondin

Isolated thermolysin-generated fragments of thrombospondin from human endothelial cells (HUVEC, HUAEC, HSVEC), human fibroblasts (HOF, HFF, HSF) and human platelets (HPL) were immunodetected in a competitive radioimmunoassay using both <sup>125</sup>I-labelled whole platelet thrombospondin and a polyclonal antibody directed against platelet thrombospondin. All the proteolytic fragments of endothelial, fibroblast and platelet thrombospondins were recognized by the polyclonal antibody (Fig. 4A and B).

#### Immunological characterization of thermolysin-generated fragments of endothelial, fibroblast and platelet thrombospondins using three different monoclonal antibodies (P10, MA-I, MA-II) directed against platelet thrombospondin

Isolated thermolysin-generated fragments of thrombospondin from human endothelial cells (HUVEC, HUAEC, HSVEC), human fibroblasts (HOF, HFF, HSF) and human platelets (HPL) were also immunodetected in an enzyme-linked immunosorbent assay using three monoclonal antibodies (P10, MA-I, MA-II). Monoclonal antibodies P10, directed against an epitope located near the hemagglutinin activity domain of platelet thrombospondin [31], and MA-I, directed against a 47-kDa SDS/tryptic fragment of platelet thrombospondin [30] essentially recognized two proteolytic fragments (135–110 kDa, 98–82 kDa) of endothelial thrombospondin (Fig. 5A–C) and three (230–195, 115–100, 92–80 kDa) of fibroblast thrombospondin (Fig. 6A–C). A strong immunodetection by P10 and MA-I was only observed with the 110-kDa fragment of platelet thrombospondin (Figs 5D and 6D). Monoclonal antibody MA-II, directed against an epitope located within the heparin-binding domain of platelet thrombospondin [30], essentially recognized three proteolytic fragments of endothelial (54–47, 25–20, 18–15 kDa) and fibroblast (54–49, 27–21, 17–13 kDa)

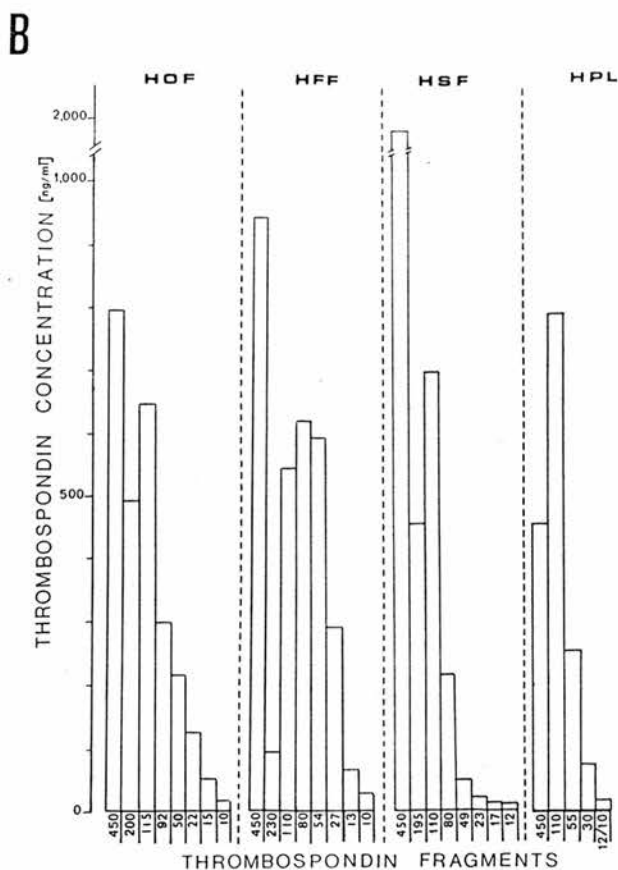
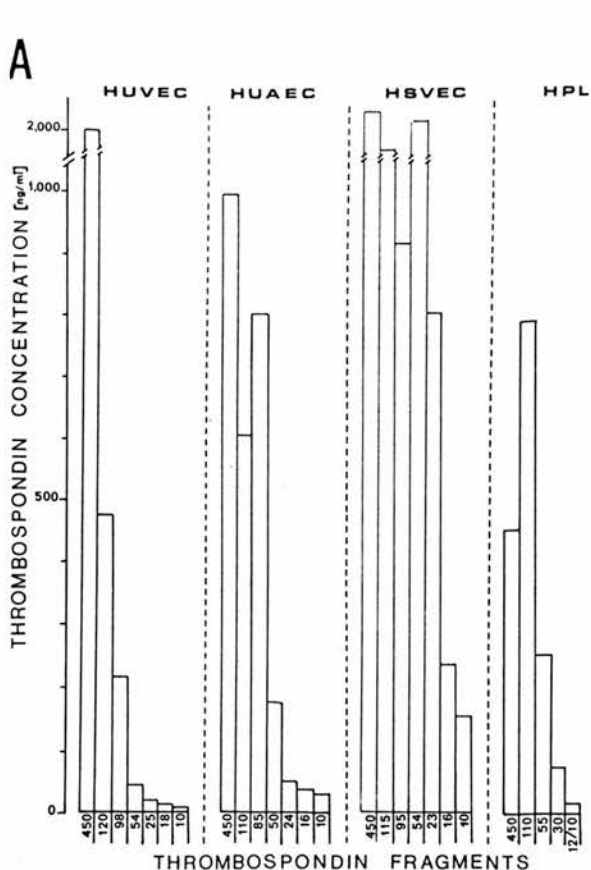


Fig. 4. Immunological characterization of thermolysin-generated fragments of endothelial, fibroblast and platelet thrombospondins using a competitive radioimmunoassay with a polyclonal antibody directed against whole platelet thrombospondin. (A) Isolated thermolysin-generated fragments of thrombospondin from human endothelial cells (HUVEC, HUAEC, HSVEC) and human platelets (HPL). (B) Isolated thermolysin-generated fragments of thrombospondin from human fibroblasts (HOF, HFF, HSF) and human platelets (HPL). Proteolytic fragments of thrombospondins were mixed with  $^{125}\text{I}$ -labelled platelet thrombospondin and a rabbit polyclonal antibody directed against whole platelet thrombospondin. After incubation overnight at  $4^\circ\text{C}$ , Sepharose-4B-coupled donkey anti-(rabbit IgG) was added and the tubes shaken for 45 min at  $20^\circ\text{C}$ . Sepharose-bound material was then separated from that remaining in the liquid phase by sedimentation of the solid phase through assay buffer containing 10% sucrose, and removal of the supernatant by aspiration. Tubes were counted in a gamma counter. The fragments are indicated by their molecular mass in kDa. The molecular mass of undigested thrombospondin is 450 kDa [9]. HPL, human platelets

thrombospondins (Figs 5A–C and 6A–C), and two fragments (50 kDa, 22 kDa) of platelet thrombospondin, different from those detected by P10 and MA-I (Figs 5D and 6D).

Analysis of the thermolysin digests of thrombospondins by SDS/polyacrylamide gel electrophoresis under reducing conditions indicated that these fragments of different molecular masses consisted of polypeptide chains held together by disulphide bonds or non-covalent associations (results not shown).

#### *Gel FPLC of human endothelial, fibroblast and platelet thrombospondin oligomers before and after thermolysin digestion*

To investigate further the structural differences observed between the three forms of thrombospondin, their respective degree of polymerization has been compared using a Superose 6 gel-permeation column.

Thrombospondin purified from human foetal vein endothelial cells (HUVEC line H55 P4) showed three major

peaks with molecular masses of 3300 kDa, 1000 and 450 kDa respectively (Fig. 7A). Concomitant with the disappearance of the thrombospondin dimer (1000 kDa) and monomer (450 kDa), thermolysin digestion of foetal vein endothelial thrombospondin generated eight peaks, one of which eluted after the total volume of the column (Fig. 7B). The proteolytic fragments corresponding to these peaks had molecular masses of 200, 130, 80, 50, 26, 14 and 11 kDa respectively. The thrombospondin heptamer (3300 kDa) was unaffected by thermolysin (Fig. 7B).

Thrombospondin purified from human foreskin fibroblasts (HFF line F34 P6) showed three major peaks with molecular masses of 3300, 900 and 450 kDa, respectively (Fig. 8A). Exposure of F34 P6 fibroblast thrombospondin to thermolysin resulted in a decrease of the thrombospondin dimer (900 kDa) and monomer (450 kDa), and the concomitant appearance of five major peaks, one of which eluted after the total volume of the column (Fig. 8B). The proteolytic fragments corresponding to these peaks had molecular masses of 83, 54, 27, 13 and 10 kDa respectively. The thrombospondin heptamer (3300 kDa) was unaffected by thermolysin (Fig. 8B).

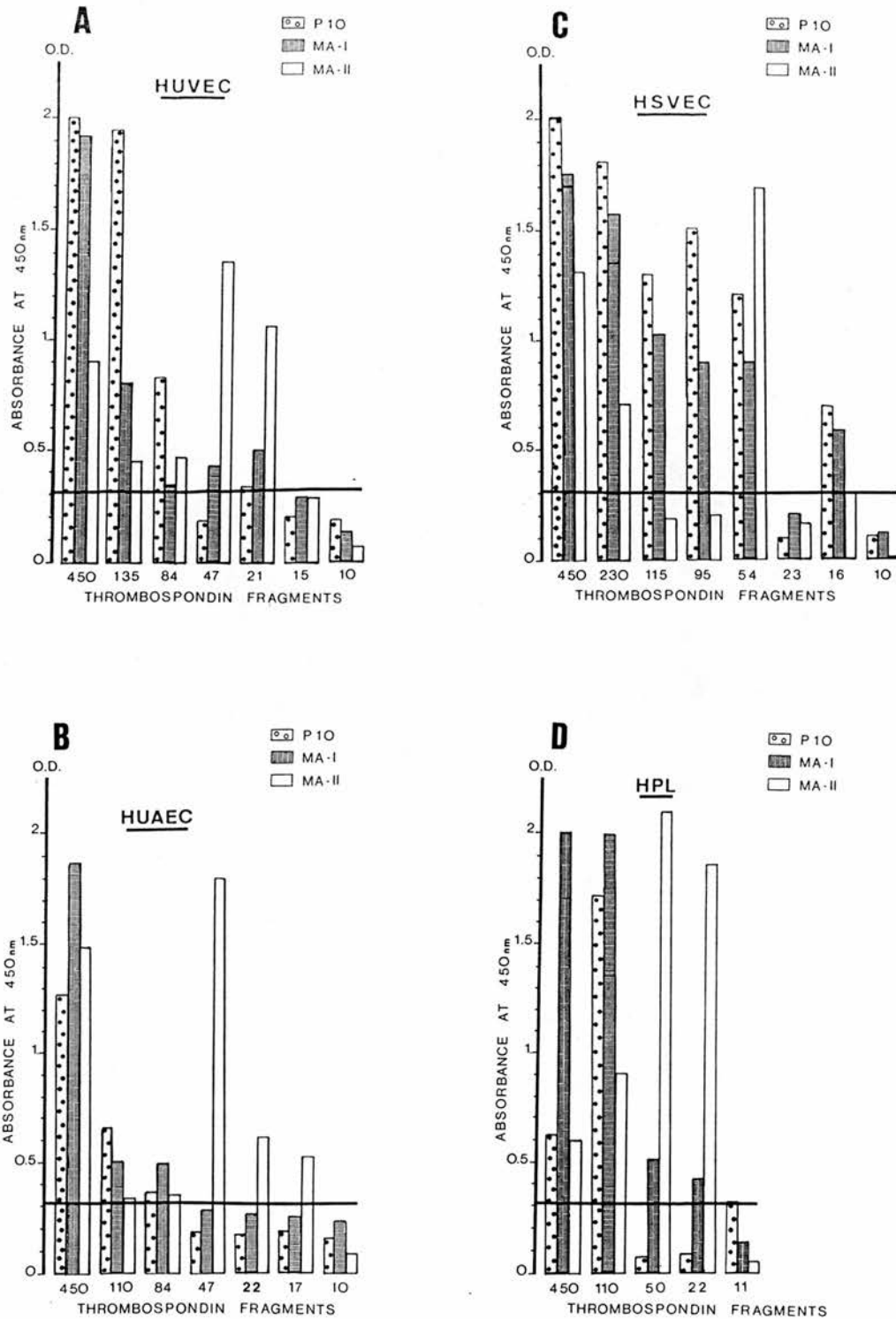


Fig. 5. Immunological characterization of thermolysin-generated fragments of endothelial and platelet thrombospondins using an enzyme-linked immunosorbent assay with three monoclonal antibodies (P10, MA-I, MA-II) directed against different epitopes of whole platelet thrombospondin. Binding of monoclonal antibodies P10, MA-I and MA-II to isolated thermolysin-generated fragments of thrombospondin purified from (A) human umbilical cord vein endothelial cells, (B) human umbilical cord artery endothelial cells, (C) human saphenous vein endothelial cells and (D) human platelets. Thermolysin-generated fragments of thrombospondins isolated from the Superose 12 gel-permeation column were adsorbed to 96-well microtitre plates by incubating 48 h at room temperature, followed by extensive washing with phosphate-buffered saline containing 0.1% (v/v) Tween 20 (washing buffer). Monoclonal antibodies P10, MA-I and MA-II were then added to the fragments bound to the microtitre plates for 1 h incubation at 37°C. After rinsing the microtitre plates with the washing buffer, goat anti-(mouse IgG) conjugated with horseradish peroxidase was added for an additional 1 h incubation at 37°C. Following washing of the microtitre plates, the substrate solution was added and the enzyme reaction was allowed to proceed for 10 min at room temperature in the darkness. Results showed the binding of monoclonal antibodies P10, MA-I and MA-II, with duplicate determinations for each proteolytic fragment. The black line showed the level of non-specific binding for the second negative control (see Experimental Procedure). Thrombospondin fragments are indicated by their molecular mass in kDa. The molecular mass of undigested thrombospondin is 450 kDa [9]



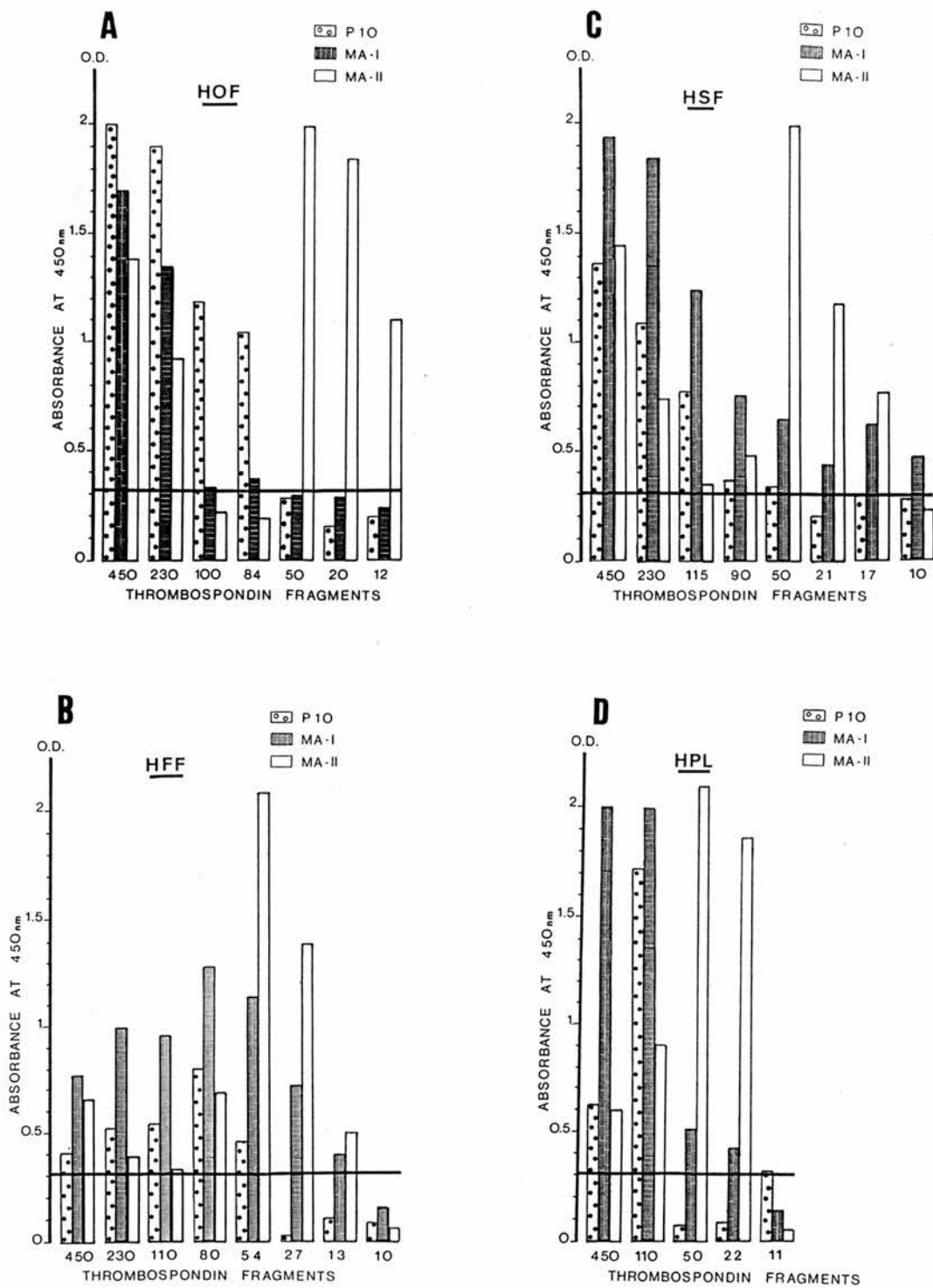


Fig. 6. Immunological characterization of thermolysin-generated fragments of fibroblast and platelet thrombospondins using an enzyme-linked immunosorbent assay with three monoclonal antibodies (P10, MA-I, MA-II) directed against different epitopes of whole platelet thrombospondin. Binding of P10, MA-I and MA-II to isolated thermolysin-generated fragments of thrombospondins purified from (A) human oral fibroblasts, (B) human foreskin fibroblasts, (C) human forearm skin fibroblasts and (D) human platelets. Experimental conditions were similar to those described in Fig. 5

Thrombospondin purified from human platelets showed four major peaks with molecular masses of 3300, 2100, 990 and 450 kDa respectively (Fig.9A). However, only three major peaks (3300, 900 and 450 kDa) were observed with another platelet thrombospondin preparation when injected onto the Superose 6 gel-permeation column under identical

experimental conditions (results not shown). Exposure of human platelet thrombospondin to thermolysin resulted in a decrease of these four oligomers of thrombospondin and the concomitant appearance of five major peaks, one of which eluted after the total volume of the column (Fig.9B). The proteolytic fragments corresponding to these peaks had mo-

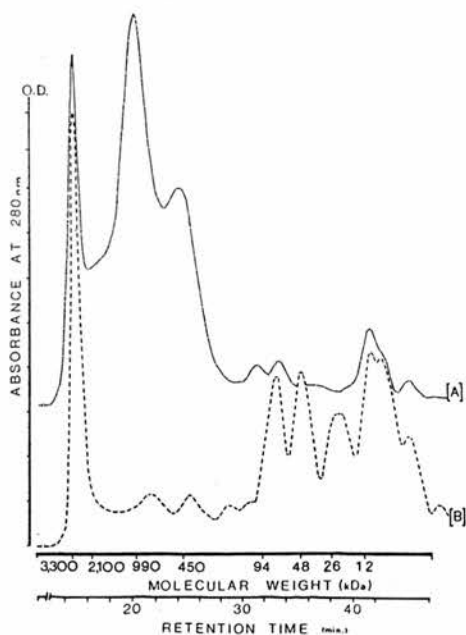


Fig. 7. Gel FPLC of human endothelial thrombospondin oligomers before and after thermolysin digestion. Thrombospondin was purified from human umbilical cord vein endothelial cells (HUVEC line P4) and an equal amount of protein (30  $\mu$ g in 0.5 ml) was then injected onto the Sepharose 6 gel-permeation column before (A) and after (B) thermolysin digestion. The thermolysin/thrombospondin ratio was 1:20 (w/w). After 90 min of incubation at 37°C, the thermolysin digestion was terminated by addition of 10 mM EDTA. The Sepharose 6 gel-permeation column connected to a FPLC system was equilibrated with 20 mM triethanolamine buffer (pH 7.4) containing 0.5 M NaCl. The flow rate was 0.5 ml/min.

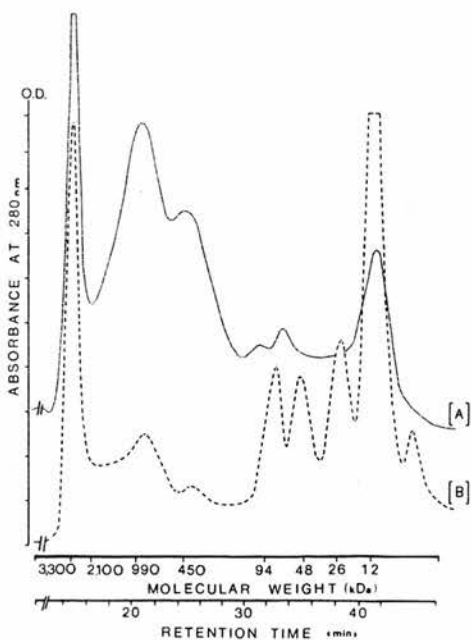


Fig. 8. Gel FPLC of human fibroblast thrombospondin oligomers before and after thermolysin digestion. Thrombospondin was purified from human foreskin fibroblasts (HFF line F34 P6) and an equal amount of protein (30  $\mu$ g in 0.5 ml) was then injected onto the Superose 6 gel-permeation column before (A) and after (B) thermolysin digestion. Experimental conditions were similar to Fig. 7.

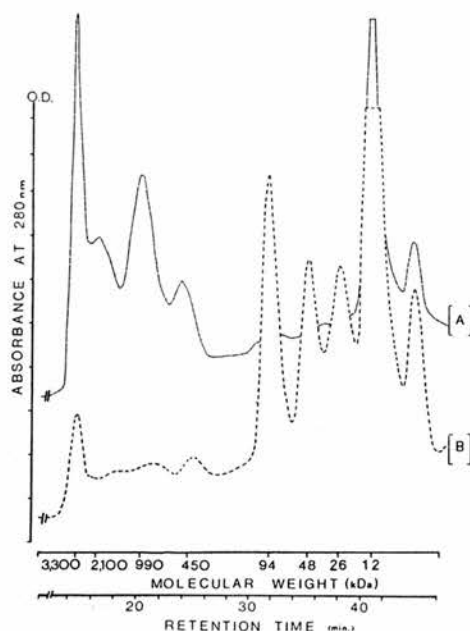


Fig. 9. Gel FPLC of human platelet thrombospondin oligomers before and after thermolysin digestion. Thrombospondin was purified from thrombin-stimulated platelets and an equal amount of protein (30  $\mu$ g in 0.5 ml) was then injected onto the Superose 6 gel-permeation column before (A) and after (B) thermolysin digestion. Experimental conditions were similar to Fig. 7.

molecular masses of 94, 48, 26 and 12 kDa respectively. When using a Superose 12 column, thermolysin digestion of the same platelet thrombospondin preparation generated five major proteolytic fragments with molecular masses of 110, 50, 22, 12 and 10 kDa respectively (results not shown).

Oligomers of the three forms of thrombospondin were all recognized by a polyclonal antibody and three monoclonal antibodies (P10, MA-I, MA-II) directed against whole platelet thrombospondin (results not shown).

## DISCUSSION

In agreement with previous studies [3, 5, 6, 25], endothelial, fibroblast and platelet thrombospondins were found to be immunologically indistinguishable since a polyclonal antibody and three monoclonal antibodies (P10, MA-I, MA-II) recognized all three forms of thrombospondin in their native state. Moreover, when assayed in a radioimmunoassay, the use of a polyclonal antibody which recognizes all the proteolytic fragments of endothelial, fibroblast and platelet thrombospondins indicates that these three forms of thrombospondin share common determinants. These results are consistent with the fact that platelet and endothelial thrombospondins have similar amino acid compositions [3]. The immunological identification by monoclonal antibodies P10, MA-I and MA-II of the thermolysin-generated fragments of thrombospondins shows that these common determinants were borne by similar fragments of endothelial, fibroblast and platelet thrombospondins. When analyzed by SDS/polyacrylamide gel electrophoresis under reducing conditions, these proteolytic fragments consisted of polypeptide chains held together by disulphide bonds or non-covalent associations, some of them being present within several proteolytic fragments (P. Clezardin, unpublished results). Such an observation has also been reported by Lawler et al.

[30] studying the structure of platelet thrombospondin after trypsin or thrombin digestion. It is, therefore, conceivable that the binding of P10, MA-I and MA-II to several proteolytic fragments could be due to the fact that these fragments are related and have arisen by successive cleavage of whole thrombospondin when using non-denaturing conditions.

Beside these identical immunological properties, endothelial and fibroblast thrombospondins are, however, structurally different from platelet thrombospondin since two fragments of endothelial thrombospondin (98–82 kDa, 18–15 kDa), equivalent to those of fibroblast thrombospondin (92–80 kDa, 17–13 kDa), are not released from platelet thrombospondin when digested with thermolysin. Using trypsin digestion we recently reported such structural differences between thrombospondins purified from platelets and from foetal vein endothelial cells [25]. However, since qualitative and quantitative differences occurred in the secretory phenotypes of foetal and adult endothelial cells [27–29], we could not exclude from our preliminary study the possibility that the structural differences observed were a result of the foetal origin of the endothelial cells. The results obtained in this study demonstrate that, whether the cells are of foetal or adult origin, a unique proteolytic pattern is obtained after thermolysin digestion of endothelial thrombospondin.

Following the observation that a high intracellular degradation of unglycosylated thrombospondin occurs in human endothelial cells when cultivated in the presence of tunicamycin, Vischer et al. [36] suggest that the carbohydrate moiety of thrombospondin does protect the glycoprotein partially against proteolysis. Since changes in the glycosylation of glycoproteins take place in human cells as they approach confluence [26] or senescence in culture [37], it was conceivable that such changes in the carbohydrate moiety of thrombospondin isolated from confluent cells could affect the enzymatic proteolysis of this glycoprotein, and thus account for the structural differences observed in our preliminary study [25]. In the present work we did not investigate the carbohydrate structures of endothelial, fibroblast and platelet thrombospondins. However, we demonstrated that the thermolysin-generated fragments of endothelial and fibroblast thrombospondins were similar when isolated from subconfluent or confluent cells. Such results suggest that changes in the glycosylation of thrombospondins isolated from confluent cells, if any, are not detected under our experimental conditions of chromatography, and thus do not account for the structural differences observed between the three forms of thrombospondin. Moreover, these results are in agreement with the fact that both thrombospondins from cultured endothelial cells and platelets have similar carbohydrate contents [36].

To investigate further the structural differences observed between endothelial, fibroblast and platelet thrombospondins, their respective degree of polymerization has been compared by Superose 6 gel-permeation chromatography under non-denaturing conditions. The three forms of thrombospondin commonly elute over a broad molecular mass range with major oligomers at 3300 kDa, 900 kDa and 450 kDa. Similar results were previously reported by Booth et al. [10] studying the degree of polymerization of human platelet thrombospondin by Sepharose 2B gel-filtration chromatography. Despite thermolysin digestion, the persistence of the 3300-kDa oligomer of endothelial or fibroblast thrombospondin indicates that the 900-kDa and 450-kDa oligomers are degraded at a protease to thrombo-

spondin ratio higher than that for platelet thrombospondin oligomers, and thus favours the release of proteolytic fragments from endothelial and fibroblast thrombospondins smaller than those released from platelet thrombospondin. Such an assumption could also explain the variability of the elution profiles of the thermolysin-generated fragments from one cell line to another since the amount of thrombospondin recovered at 3300 kDa may also vary from one cell culture to another (P. Clezardin, unpublished results). However, the fact that the 3300-kDa oligomer of platelet thrombospondin is degraded by thermolysin whereas its endothelial or fibroblast counterpart is not degraded, suggests differential conformational properties between platelet and the two other forms of thrombospondin. McKeown-Longo et al. [38] reported that the addition of purified  $^{125}\text{I}$ -labelled platelet thrombospondin to human lung fibroblasts resulted in the degradation after 3 h of 60% of the radiolabelled thrombospondin molecule. On the other hand, in pulse-chase experiments designed to study the endogenous metabolism of thrombospondin in human skin fibroblasts, Mumby et al. [39] observed that the level of radiolabelled fibroblast thrombospondin secreted into the medium remained constant from 1 h to 12 h of chase time. The authors [39] related these conflicting results between the two studies to differences in cellular recognition of platelet versus fibroblast thrombospondin, or between thrombospondin labelled with  $^{125}\text{I}$  by the chloramine T method versus metabolically labelled thrombospondin. Such differences could also result from an increased resistance to proteolysis of fibroblast thrombospondin due to conformational properties different from those of platelet thrombospondin, as suggested by this study. Further work is in progress to clarify the role of the conformation of the thrombospondin molecule in the polymorphism of endothelial and fibroblast thrombospondins versus platelet thrombospondin.

*Note.* While this manuscript was under review, Dardik et al. [40] reported, as an abstract, that the proteolytic pattern of endothelial thrombospondin was similar but not identical to that of platelet thrombospondin.

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# Thrombospondin in Milk, Other Breast Secretions, and Breast Tissue

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and DAVID A. PRATT, M.Sc.

The trimeric glycoprotein thrombospondin was first isolated from the extracellular medium after thrombin stimulation of platelets,<sup>1</sup> but has since been shown to be synthesized and secreted by a wide range of cell types, including endothelial cells,<sup>2,3</sup> smooth muscle cells,<sup>4</sup> fibroblasts,<sup>5</sup> and monocytes.<sup>6</sup> In 1985 we reported that thrombospondin is also present in high concentrations in milk<sup>7</sup> and in some breast cyst fluids,<sup>8</sup> where the low levels of the other platelet  $\alpha$ -granule proteins  $\beta$ -thromboglobulin and platelet factor 4 clearly indicate a nonplatelet source for thrombospondin.

However, the cell type responsible for the production of thrombospondin in the mammary gland has not been identified, nor is there any indication of the function of thrombospondin in this context. This article describes two approaches to these questions. In one, production of thrombospondin during the initiation of lactation is examined and the distribution of thrombospondin in different milk fractions defined. Thrombospondin was also purified from goat colostrum and its structure compared with the protein isolated from other sources. The second approach involves the measurement of thrombospondin in a range of breast secretions and breast tissue samples in an attempt to analyze the conditions under which it is synthesized and secreted.

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## MATERIALS AND METHODS

### Samples

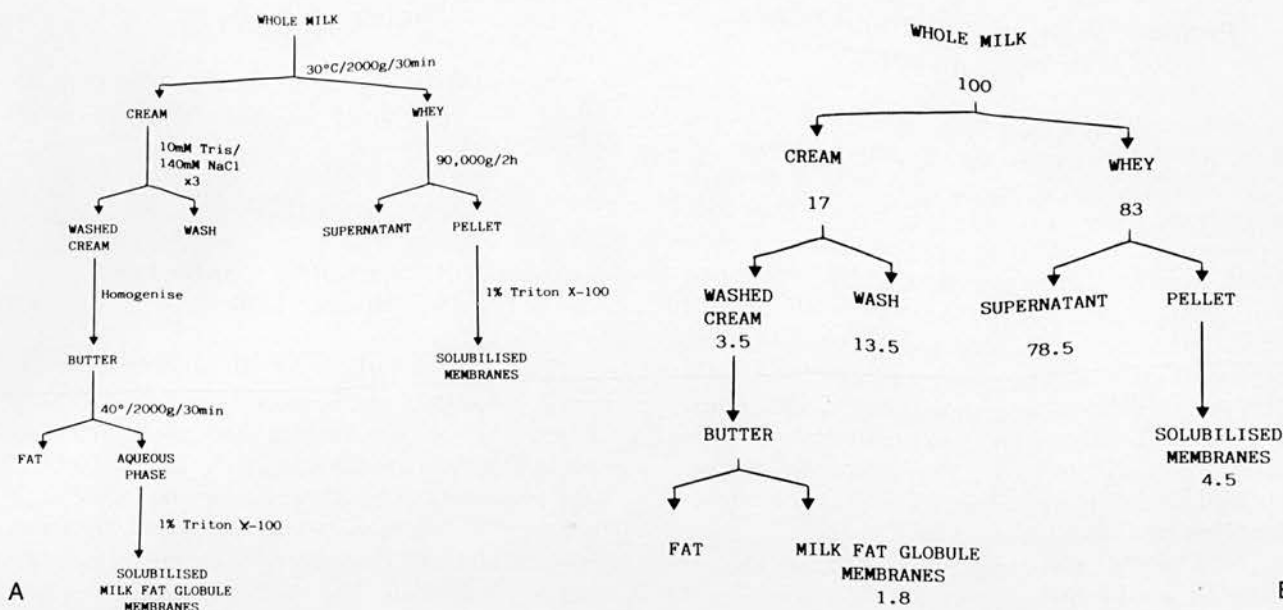
Human colostrum and milk were obtained by manual expression and other breast secretions by nipple aspiration, with the exception of fluid from patients with galactorrhea, which were spontaneous discharges. Breast cyst fluids were obtained by needle aspiration, then centrifuged at  $600 \times g$  for 10 minutes and the supernatants stored at  $-20^{\circ}\text{C}$  until assayed.

Unpasteurized cow's milk was supplied by Jean Robb, Department of Microbiology, University of Edinburgh, and goat colostrum by Dr. Ken Brown, ARC Institute of Animal Physiology, Cambridge, UK.

Breast tissue samples (1 g wet weight) were homogenized in 5 ml 10 mM Tris buffer pH 8.0 containing 1.5 mM ethylene diaminetetraacetic acid (EDTA) and 0.25 M sucrose, using a Silverson homogenizer. They were centrifuged at  $105,000 \times g$  at  $4^{\circ}\text{C}$  for 1 hour, and the resulting supernatant was stored at  $-20^{\circ}\text{C}$ .

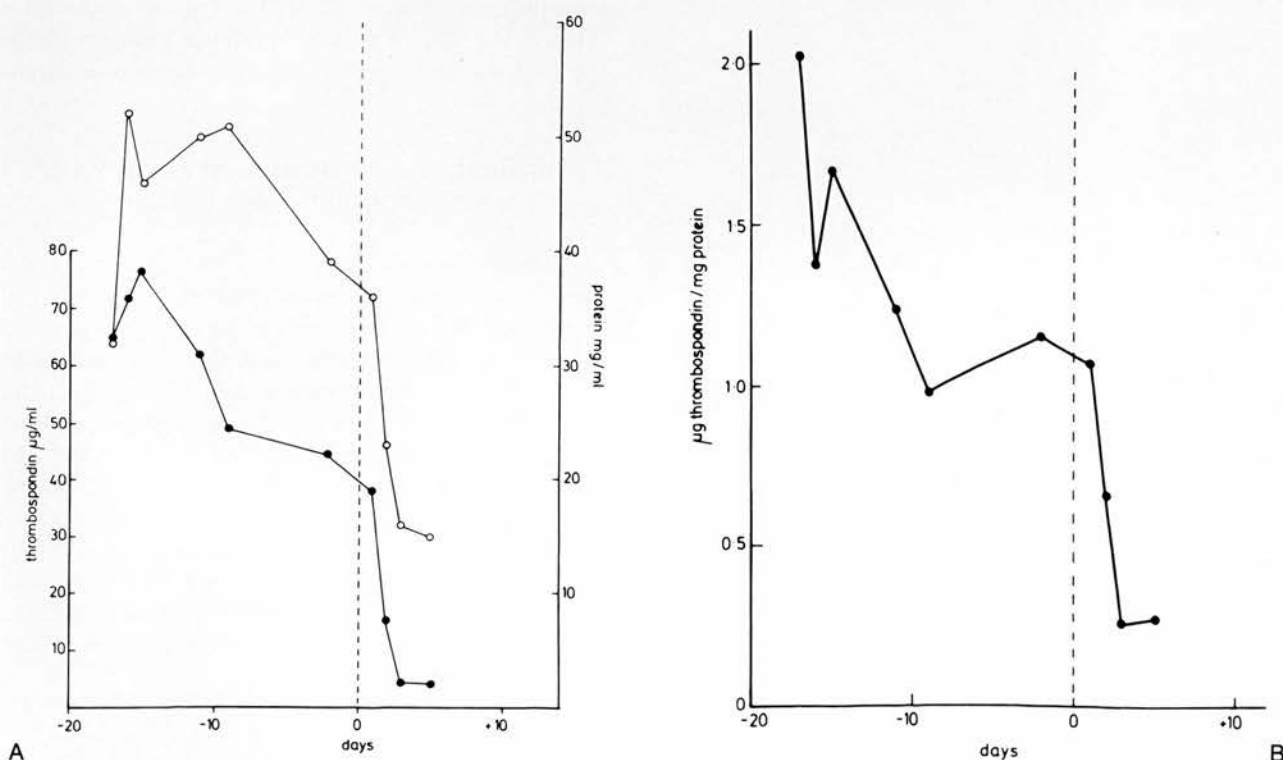
### Fractionation of Milk

Fresh unpasteurized bovine milk (1 liter) was fractionated by a modification of the methods of Mather<sup>9</sup> and Shimizu and Yamauchi,<sup>10</sup> which is summarized in Figure 1a. The milk was warmed to  $30^{\circ}\text{C}$  and centrifuged at  $2000 \times g$  for 30 min, maintaining the temperature at  $30^{\circ}\text{C}$ . The raw cream (top layer) was removed and washed three times at  $30^{\circ}\text{C}$  with 3 to 5 volumes of 10 mM Tris buffer, pH 7.2, containing 140 mM NaCl. Washed



cream was cooled and homogenized with an equal volume of the buffer to give butter. The aqueous phase was recovered by centrifugation at  $2000 \times g$  for 30 minutes after melting the fat granules at  $40^{\circ}\text{C}$ . Milk fat globule membranes in the aqueous phase were solubilized in 1% Triton X-100. The

whey fraction was centrifuged at  $90,000 \times g$  for 2 hours at  $4^{\circ}\text{C}$  to remove contaminating membranes and residual lipid, and the pellet was solubilized in 1% Triton X-100. The volumes of all fractions were measured and samples stored at  $-20^{\circ}\text{C}$  until assayed.



**FIG. 2. Thrombospondin (●) and total protein (○) concentrations (A) and relative thrombospondin concentrations (B) in human colostrum and milk during the initiation of lactation. Parturition occurred at day 0 (vertical line).**

### Purification and Structural Analysis of Milk Thrombospondin

Whey was prepared from 300 ml goat colostrum as previously described, after addition of NaCl to a final concentration of 0.35 M. Polyvinylpyrrolidone (PVP) was then added to a final concentration of 3% (w/v) before incubation at 20°C for 15 minutes. Centrifugation at  $35,000 \times g$  for 60 min removed the complex of PVP with residual lipid. The supernatant was applied to a heparin-Sepharose column, which was washed with 15 mM Tris buffer pH 7.4, containing 2 mM  $\text{CaCl}_2$  and 0.35 M NaCl before elution of the thrombospondin at 0.55 M NaCl (Fig. 3). The eluate was further purified on an anion exchange Mono Q column, as described by Clezardin et al.<sup>11</sup>

The product was compared with human platelet thrombospondin by analysis on a Superose 12 HR 10/30 gel filtration column<sup>12</sup> both before and after digestion with thermolysin (Sigma) at a ratio of 1:20 (w/w) for 90 minutes at 37°C.

### Assay of Thrombospondin

Thrombospondin was measured by radioimmunoassay,<sup>13</sup> using a polyclonal rabbit antiserum that detects human, bovine, and goat thrombospondins.

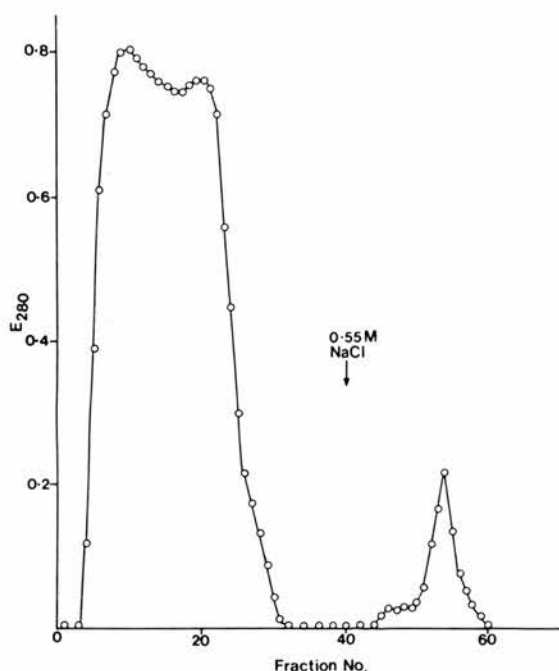


FIG. 3. Affinity purification of thrombospondin from colostrum on heparin-Sepharose. After defatting as described in the text, 300 ml goat colostrum was applied to a heparin-Sepharose column in 0.35 M NaCl. The peak eluted with 0.55 M NaCl contained all the thrombospondin applied.

### Statistical Analysis

Statistical analysis of differences between populations was performed by the Wilcoxon-Mann-Whitney rank sum test.

## RESULTS

### Thrombospondin Content of Human Milk

The concentration of thrombospondin in human colostrum and in milk during the initiation of lactation is compared with the total protein content in Figure 2a, which covers the period from 17 days prepartum to 5 days postpartum. Colostrum contained as much as 70 to 80  $\mu\text{g}/\text{ml}$  thrombospondin, and this dropped to 4  $\mu\text{g}/\text{ml}$  as lactation became established. The ratio of thrombospondin to total protein over this period (Figure 2b) declined almost tenfold from 2 to 0.25  $\mu\text{g}$  thrombospondin/mg protein.

### Distribution of Thrombospondin in Milk

When fresh unpasteurized cow's milk was fractionated according to the procedure summarized in Figure 1a, more than 90% of the total thrombospondin content was recovered in the aqueous phase (Figure 1b). There was no evidence that thrombospondin was a milk fat globule membrane glycoprotein.

### Purification and Structural Analysis of Thrombospondin from Colostrum

Thrombospondin was purified from goat colostrum. The lipid content of the starting material resulted in major problems for both affinity and gel filtration chromatography, and this was minimized by the use of NaCl and PVP. Goat colostrum thrombospondin bound to heparin-Sepharose in the presence of 0.35 M NaCl and eluted in 0.55 M NaCl (Fig. 3). After further purification on an anion exchange Mono Q column, the product was gel filtered on a Superose 12 column. It yielded a major peak at 450 kd coincident with that obtained with human platelet thrombospondin (Fig. 4), but the colostrum protein contained more fragments of low molecular weight (Table 1).

After thermolysin digestion of thrombospondin from the two sources followed by gel filtration, the fragmentation patterns were found to be completely different (Fig. 5). The higher molecular weight deg-

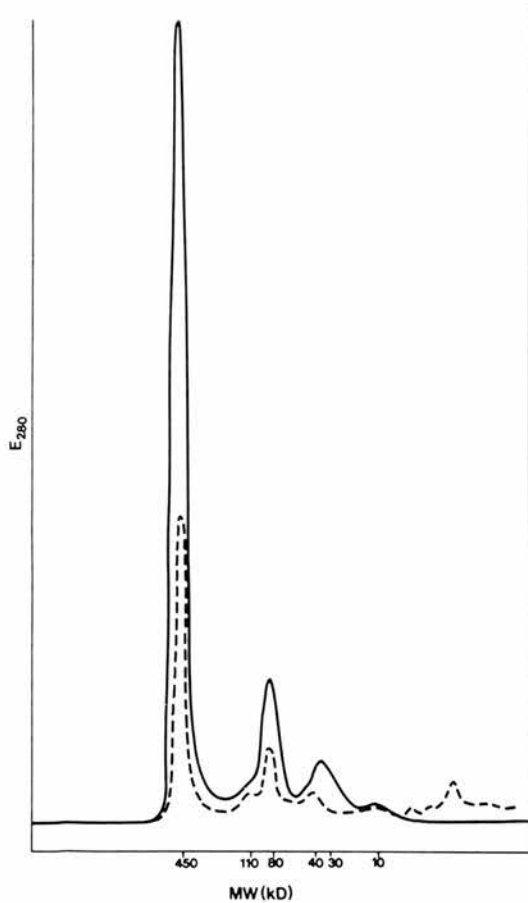


FIG. 4. Gel filtration of thrombospondin from goat colostrum (-----) and human platelets (—) on Superose 12.

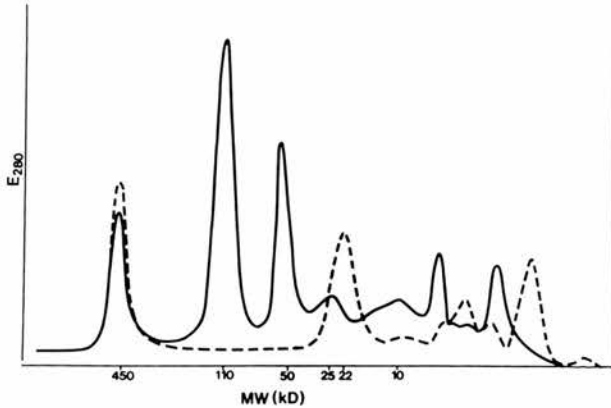


FIG. 5. Gel filtration of thermolysin digests of thrombospondin from goat colostrum (-----) and human platelets (—) on Superose 12.

radiation products of human platelet thrombospondin were absent from the digest of goat colostrum thrombospondin, which contained several very small fragments. This pattern is unique among the thrombospondins from different sources examined to date (Table 2).

### Thrombospondin in Other Breast Secretions

In the absence of lactation, small volumes of fluid can be secreted by the breasts of healthy women as well as by those with fibrocystic disease and breast cancer. In galactorrhea the flow increases considerably. All these secretions contained thrombospondin in the range 1 to 145  $\mu\text{g}/\text{ml}$ , with a median of 23  $\mu\text{g}/\text{ml}$  (Fig. 6), much the same as that in milk and colostrum, and there was no significant difference between concentrations in the different types of secretion. Fluids from the type of breast cyst containing high levels of  $\text{Na}^+$  contained concentrations of thrombospondin in the same range, (median, 7.6; range, 0.96–51.1  $\mu\text{g}/\text{ml}$ ) although they were significantly lower ( $p < 0.001$ ) than the levels in breast secretions. Thrombospondin levels in fluids from cysts with comparatively higher  $\text{K}^+$  and lower  $\text{Na}^+$  were much lower (median, 1.15; range, 0.06–5.6  $\mu\text{g}/\text{ml}$ ), and very significantly different ( $p < 10^{-6}$ ) from those in any other breast fluids.

### Thrombospondin in Breast Tissue

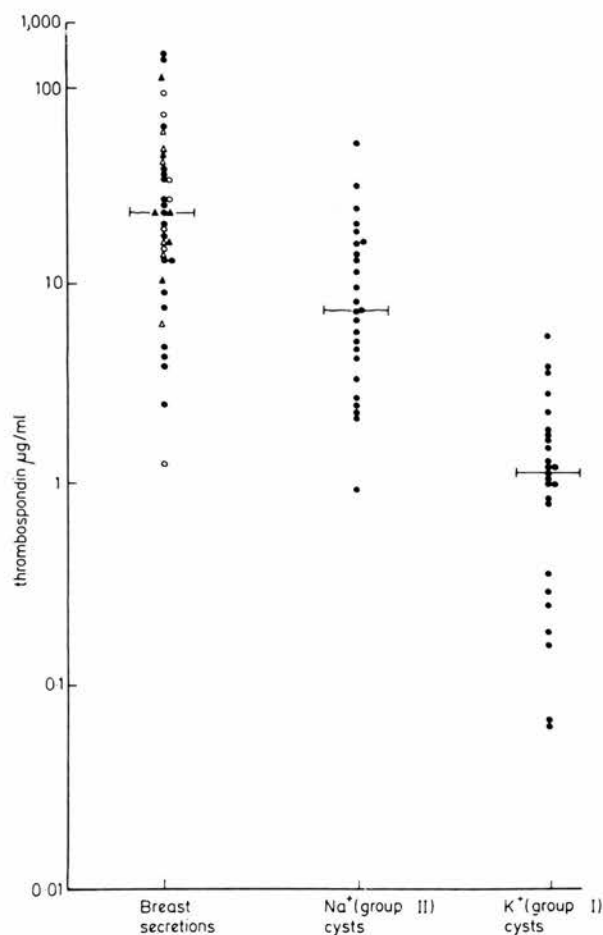
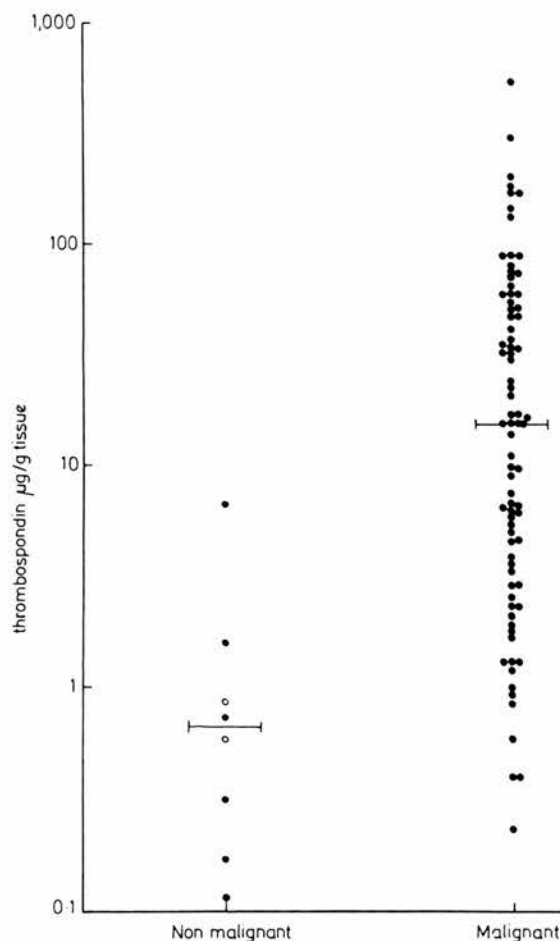
Few samples of normal breast tissue, usually noninvolved tissue at the periphery of a malignancy, have been obtained as yet. However, most of these contained less than 1  $\mu\text{g}$  thrombospondin/gm wet weight of tissue, as did the two samples of benign mammary dysplasia examined (Fig. 7). By contrast, 90% of the cytosols from malignant breast tissue contained more than this concentration of thrombospondin (median, 15.5; range, 0.23–550  $\mu\text{g}/\text{gm}$ ) and the difference between the thrombospondin contents of malignant and nonmalignant tissues (median, 0.66; range, 0.11–6.7  $\mu\text{g}/\text{gm}$ ) was very highly significant ( $p < 10^{-6}$ ).

TABLE 1. Fragments (% Total) in Freshly Isolated Thrombospondin

Origin	Fragments (kd)							
	450	110	80	30-40	10	<10	<10	<10
Human platelets	75.4	2.3	13.4	7.5	1.4	—	—	—
Goat colostrum	51.4	7.2	12.6	9.6	2.5	4.0	3.8	8.9

**TABLE 2. Thermolysin-Generated Fragments of Thrombospondins from Different Sources**

Origin		Fragments (kd)							
Human platelets	450	110	50	25	10				
Human endothelial cells <sup>12</sup>	450	120	85	50	25	16	10		
Human fibroblasts <sup>14</sup>	450	230	110	85	50	25	16	10	
Goat colostrum	450				22	10	<10	<10	<10

**FIG. 6. Thrombospondin concentrations in human breast secretions and breast cyst fluids.** Breast secretions were from healthy women (●), and patients with breast cancer (○), fibrocystic disease (Δ) and galactorrhea (▲).**FIG. 7. Thrombospondin concentrations in normal breast tissue and breast cancer.** Included with the normal values (●) are two samples from benign dysplasia (○).

## DISCUSSION

Thrombospondin is not a major milk protein in that it constitutes only 0.2% of the total protein of colostrum and 0.02% of that of milk. Serum proteins also include 0.02% thrombospondin, but in body fluids in vivo the content is much lower; in plasma, for instance, thrombospondin makes up only 0.00015% of the total protein. Therefore in relative terms the concentration of thrombospondin

in milk and other breast secretions is high, and this raises the question of its function.

Milk fat, the melting point of which is lower than body temperature, is dispersed in droplets that are prevented from coalescing by a membrane consisting of phospholipid-protein complexes and rich in glycoproteins.<sup>9</sup> Thrombospondin was not, however, associated with the milk fat globule membrane but was localized in soluble form in the aqueous phase.



Apart from the necessity for removal of lipid from the starting material, thrombospondin could be purified from colostrum by the techniques already developed for platelet-derived thrombospondin. Its affinities for heparin and anion exchange materials were the same as when the protein was purified from other sources. It did, however, contain smaller fragments than platelet-derived thrombospondin. These may have been the products of uninhibited protease activity in the colostrum, since very small fragments were also generated by digestion with thermolysin. The major differences between the thermolysin digestion products of colostrum thrombospondin and thrombospondins from other sources indicate differences in amino acid sequence. These may, however, be species- rather than source-specific, since goat colostrum was used in the absence of sufficient quantities of the human material. Further analyses must be performed on thrombospondin from human milk.

The pattern of thrombospondin secretion during the initiation of lactation in man was very similar to that already found in the goat, but the concentration was lower.<sup>7</sup> Although the absolute concentrations of all proteins decrease during the transition from colostrum to true milk production, the relative concentrations of specific milk proteins, such as  $\alpha$ -lactalbumin, increase,<sup>15</sup> whereas that of thrombospondin decreased tenfold, resembling the pattern of IgA production.<sup>15</sup> This implies that synthesis of thrombospondin and the specific milk proteins are under separate control. Tissue plasminogen activator (tPA) also occurs in milk in high concentrations compared with blood, and its pattern of production appears to resemble that of thrombospondin.<sup>16</sup>

A further similarity between tPA and thrombospondin in the breast is the increased levels associated with malignancy. Some breast cancer cytosols contained thrombospondin concentrations in the same range as normal tissue, but 65% were above the normal range and in some the thrombospondin levels were two orders of magnitude greater than those in normal breast. Benign dysplasias, by contrast, contained normal concentrations of thrombospondin. We are currently attempting to establish the differences between cancer tissues with high and low thrombospondin levels. The production of tPA by breast cancer cells in culture is under hormonal regulation,<sup>17</sup> and if the same is true of thrombospondin, its concentration may be related to the presence or absence of hormone receptors on the malignant cells.

All breast secretions contained thrombospon-

din in the concentration range found in colostrum and milk and apparently reflect the same mechanism of synthesis and secretion. Thrombospondin levels in breast cyst fluids, however, varied with the type of cyst. Those with higher thrombospondin concentrations, the Na<sup>+</sup> or group II cysts, are invariably lined by thin attenuated epithelial cells,<sup>18</sup> and it has been inferred that their contents accumulate in a passive manner, possibly as a transudate from blood. It is quite clear that this cannot be the case for thrombospondin, which is undoubtedly being synthesized by the cells normally responsible for its production in the breast. K<sup>+</sup> or group I cysts are usually lined by active apocrine cells,<sup>18</sup> and since the thrombospondin concentration in fluids from these cysts is very low, it is unlikely that it is synthesized by apocrine cells. This conclusion is supported by our finding (unpublished results) that thrombospondin levels in saliva (median, 144; range, 94–157 ng/ml) are within the normal plasma range.

## SUMMARY

Colostrum and milk contained high concentrations of thrombospondin, although the concentration relative to total protein content decreased as lactation was established. Thrombospondin occurred in the aqueous phase of milk rather than as a component of the milk fat globule membrane. It could be purified from colostrum using established procedures after removal of lipid from the starting material. The intact protein had a molecular weight of 450 kD, but the product contained small peptides, perhaps as a result of proteolytic activity in the colostrum. Thrombospondin from goat colostrum displayed a different proteolytic fragmentation pattern from thrombospondins isolated from three human sources, but this could be a species- rather than tissue-specific difference.

Breast cancer cytosols contained significantly more thrombospondin than cytosols from normal tissue or benign dysplasias. Thrombospondin levels in a variety of breast secretions all fell within the range found in colostrum and milk, as did the fluids from Na<sup>+</sup> (group II) breast cysts. K<sup>+</sup> (group I) cysts, however, contained fluids with low thrombospondin concentrations, eliminating apocrine cells as the source of thrombospondin in the breast.

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# Do Extra-Platelet Sources Contribute to the Plasma Level of Thrombospondin?

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## Key words

Platelet – Thrombospondin –  $\beta$ -thromboglobulin – Marrow hypoplasia

## Summary

Thrombospondin, a trimeric glycoprotein contained in the platelet  $\alpha$ -granules, has been proposed as a marker of *in vivo* platelet activation. However, it is also synthesised by a range of other cells. The extraplatelet contribution to plasma levels of thrombospondin was therefore estimated by investigating the relationship between plasma thrombospondin levels and platelet count in samples from profoundly thrombocytopenic patients with marrow hypoplasia, using the platelet-specific  $\alpha$ -granule protein  $\beta$ -thromboglobulin as control. Serum concentrations of both proteins were highly correlated with platelet count, but while plasma  $\beta$ -thromboglobulin levels and platelet count also correlated, there was no relationship between the number of platelets and thrombospondin concentrations in plasma. Serial sampling of patients recovering from bone marrow depression indicated that plasma thrombospondin contributed by platelets is superimposed on a background concentration of at least 50 ng/ml, probably derived from a non-platelet source, and plasma thrombospondin levels do not simply reflect platelet release.

## Introduction

Thrombospondin, a trimeric glycoprotein of 460,000 kD, was originally identified as a platelet-release protein (1). Dawes et al. (2) showed that its half-life in man is considerably longer than the other  $\alpha$ -granule proteins,  $\beta$ -thromboglobulin ( $\beta$ -TG) and platelet factor 4 (PF4), and suggested that its concentration in plasma may therefore be a useful indicator of *in vivo* platelet activation. However, Lane et al. (3) compared the concentrations of these three proteins in the plasma of patients with myeloproliferative disorders, diabetes, liver disease and renal failure as well as a group undergoing total hip replacement, and concluded that in conditions which were not complicated by reduced clearance  $\beta$ -TG was a more sensitive marker of platelet activation than thrombospondin. Trzeciak et al. (4) also had difficulty relating plasma concentrations of thrombospondin to platelet release in clinical situations.

It is now recognized that thrombospondin is not a platelet-specific protein, but can be synthesised *in vitro* by a range of cell types including monocytes (5), fibroblasts (6), smooth muscle cells (7) and endothelial cells (8). Thus, although nothing is known of the rates at which these cells secrete thrombospondin *in vivo*, it is possible that platelets may contribute only part of that circulating in plasma. In this case plasma concentrations of thrombospondin would not necessarily be related to platelet release, which would explain the problems experienced in using them as markers of *in vivo* platelet activation.

The contribution of platelets to plasma concentrations of thrombospondin has been studied in thrombocytopenic patients by relating plasma levels of thrombospondin and of the platelet-specific protein  $\beta$ -TG (9) to their platelet counts. Normal plasma concentrations of thrombospondin were frequently present in the absence of detectable levels of  $\beta$ -TG, clearly indicating that much of the thrombospondin present in plasma can originate from sources other than the platelets.

## Patients and Methods

### Patients

Details of patients are given in Table 1. Individual samples were taken from 20 patients with profound marrow hypoplasia, usually following chemotherapy, and serial samples from three of these during recovery from severe bone marrow depression. In all cases chemotherapy was followed by severe peripheral pancytopenia attributable to the marrow hypoplasia. Chemotherapy in most cases was given according to the Medical Research Council protocols for acute myeloblastic leukaemia and acute lymphoblastic leukaemia, and the British National Lymphoma Institute protocol for resistant non-Hodgkin's Lymphoma. Patients were sampled prior to any platelet transfusion.

### Blood Samples

To obtain plasma samples, venous blood (2.7 ml) was collected by clean venepuncture into 0.3 ml of ice-cold platelet release inhibitor solution (ETP) containing 78 mM EDTA, 10 mM theophylline and 0.33  $\mu$ g/ml prostaglandin E<sub>1</sub> (10). All necessary precautions were taken to minimise *ex vivo* platelet release.

Serum was prepared by rapid centrifugation of whole blood after incubation in glass tubes for 30 minutes at 37° C. All samples were stored at –80° C before assay.

### Assays

Concentrations of thrombospondin and  $\beta$ -TG were measured using the radioimmunoassays described by Dawes et al. (2) and Bolton et al. (11) respectively.

## Results

The relationships between platelet count and plasma concentrations of  $\beta$ -TG and thrombospondin in these patients are illustrated in Fig. 1. When the platelet count was less than 10% of normal values, plasma  $\beta$ -TG levels were well below the normal range (20.1–68.5 ng/ml) reported by Dawes et al. (2) in the majority of cases. Thrombospondin concentrations, however, were mainly within or above the normal range (57.5–215.6 ng/ml). While there was reasonable correlation between platelet count and plasma  $\beta$ -TG concentration ( $r = 0.67$ ; Fig. 1A), there was no relationship between platelet count and plasma thrombospondin ( $r = -0.08$ ; Fig. 1B).

These results contrast with the serum concentrations of  $\beta$ -TG and thrombospondin (Fig. 2), which were both depressed to a similar extent as the platelet count and were highly correlated with it ( $\beta$ -TG,  $r = 0.89$ ; Fig. 2A; thrombospondin,  $r = 0.97$ ; Fig. 2B).

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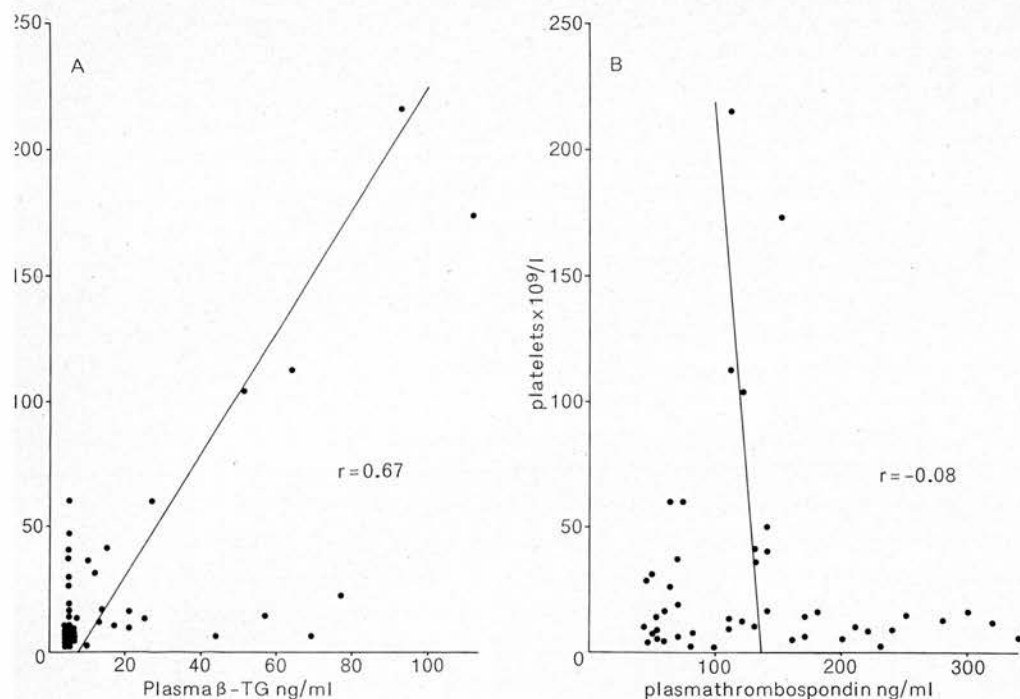


Fig. 1 The relationship between platelet count and plasma concentrations of (A)  $\beta$ -TG and (B) thrombospondin in patients with, and during recovery from, severe bone marrow depression

Table 1 Details of thrombocytopenic patients

Patient	Age	Sex	Diagnosis	Chemotherapy	Days post chemotherapy sampled
1	38	M	Resistant Hodgkin's Disease	BNLI	10
2	16	F	ALL	UKALLX	12
3	25	F	Resistant non-Hodgkin's Lymphoma	BNLI	9
4	38	M	Aplastic anaemia	—	—
5	46	F	AML (remission re-induction)	ME	11
6	78	M	Myelofibrosis	—	—
7	32	F	Resistant non-Hodgkin's Lymphoma	BNLI	3
8	63	M	Waldenstrom's macroglobulinaemia	CHOP	5
9	32	M	AML (remission consolidation)	MAZE	19 and 11 to 24
10	88	F	RAEB transformed to AML	—	—
11	79	F	ALL	UKALLX	12
12	53	M	AML (remission consolidation)	ME	18
13	49	F	AML (remission induction)	DAT	-6 to 41
14	62	F	AML (remission consolidation)	DAT MAZE	14 -1 to 28
15	57	F	PRV transformed to severe myelodysplasia	Hydroxyurea	14
16	64	M	Myeloma in escape	Melphalan (2 mg/kg)	24
17	29	M	AML (remission reinduction)	MAT	12
18	74	F	RAEB transformed to AML	Etoposide	27
19	62	F	CML	Etoposide	7
20	76	M	Hypoplastic AML	Cytarabine	42

BNLI: Cyclophosphamide, Mesna, BCNU, Cytarabine, Etoposide; CHOP: Cyclophosphamide, Doxorubicin, Vincristine, Prednisolone; DAT: Daunorubicin, Cytarabine, Thioguanine; MAZE: Amsacrine, Azacytidine, Etoposide; ME: Amsacrine, Etoposide; MAT: Amsacrine, Cytarabine, Thioguanine; UKALLX: Daunorubicin, Cytarabine, Etoposide, Thioguanine, Vincristine

Fig. 3 illustrates the time course of changes in platelet count and plasma (Fig. 3A) and serum (Fig. 3B) concentrations of  $\beta$ -TG and thrombospondin in one patient during recovery from marrow hypoplasia; similar patterns were obtained with the other two studied. Serum concentrations of the two proteins clearly followed the platelet count to a minimum concentration which reflected that of plasma. The plasma content of  $\beta$ -TG similarly rose and fell with the platelet count, though measurement of low values was limited by the sensitivity of the assay (5 ng/ml). While there was some variation in plasma thrombospondin concentra-

tion which did reflect changes in platelet count, the platelets fell by 25-fold and thrombospondin by only 2-fold, never measuring less than 50 ng/ml.

## Discussion

Throughout these studies, serum concentrations of both  $\beta$ -TG and thrombospondin correlated closely with the platelet count. This result was expected, for both are released from the platelet  $\alpha$ -granules during clotting.

Fig. 2 The relationship between platelet count and serum concentrations of (A)  $\beta$ -TG and (B) thrombospondin in patients with, during recovery from, severe marrow depression

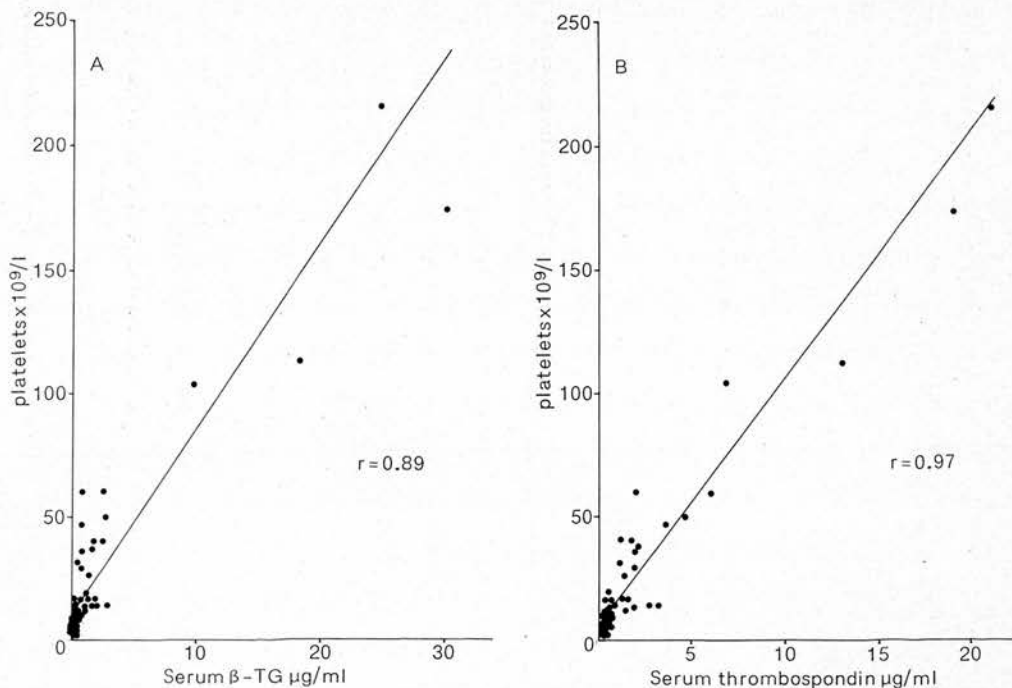
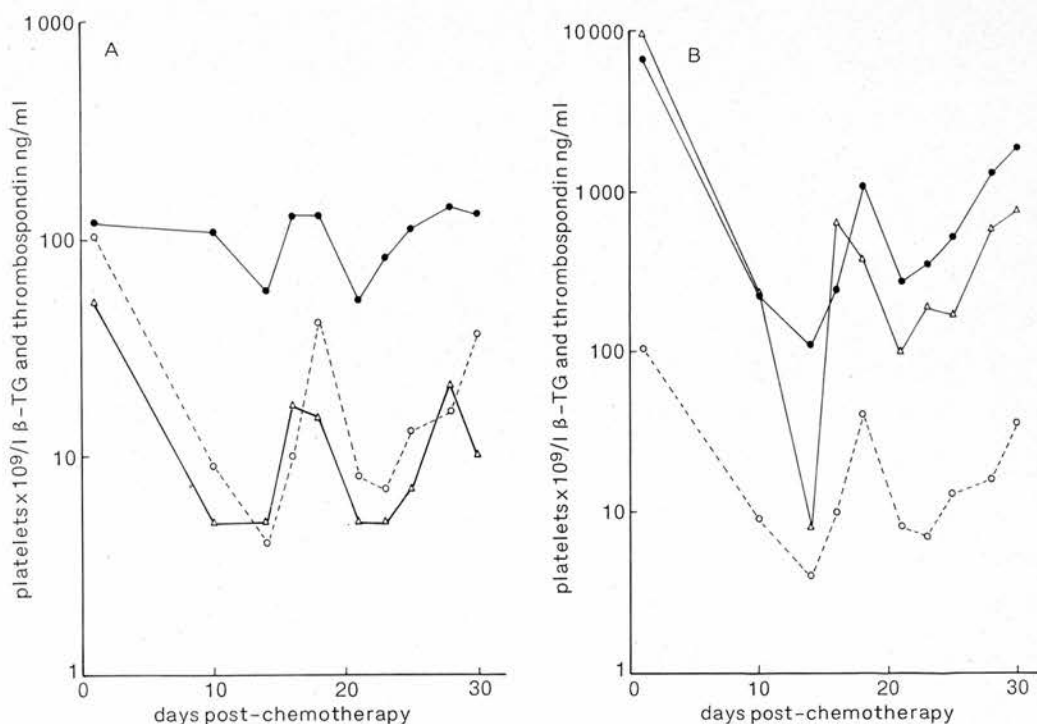


Fig. 3 Platelet count (○—○) and concentrations of  $\beta$ -TG (△—△) and thrombospondin (●—●) in serial plasma (A) and serum (B) samples from a single patient



The relationship between plasma concentrations of the two proteins and platelet count is, however, more complex. Although there was a correlation between plasma  $\beta$ -TG levels and platelet count, in 7 of the samples  $\beta$ -TG concentrations were within or even slightly above the normal range though the platelet counts were 10% or less of normal, and  $\beta$ -TG has never been identified in any cell other than platelets and megakaryocytes. As the half-life of  $\beta$ -TG in plasma is about 100 min (12), these levels cannot represent residual  $\beta$ -TG persisting from prior platelet transfusions, and as the serum levels were those expected from the

platelet counts, they cannot be accounted for by a high platelet  $\beta$ -TG content in these patients. They could, however, reflect release from defective megakaryocytes, which appears to occur in the grey platelet syndrome where plasma levels of  $\beta$ -TG are elevated (13) although those of TSP and PF4 are within the normal range (14). The possibility that the lack of correspondence between platelet count and plasma  $\beta$ -TG concentration could indicate an underlying complication of pathophysiological importance is currently being investigated. It must be emphasised, however, that pharmacokinetic factors are important modulators

the relationship between plasma  $\beta$ -TG levels and platelet count. It is clearly illustrated by the consecutive sampling of single patients that this is essentially a dynamic situation and under such circumstances platelet count and plasma  $\beta$ -TG content cannot relate perfectly as the kinetics of production and clearance of the two are not identical.

No relationship was demonstrable between plasma thrombospondin concentrations and platelet count, and although consecutive sampling showed a tendency for the two to increase and decrease together, this pattern was superimposed on a background plasma thrombospondin concentration which never fell below 50 ng/ml. In the example illustrated (Fig. 3A) the platelet count dropped 25-fold and the plasma thrombospondin only 2-fold.

Thus plasma thrombospondin concentrations probably include a major contribution from extra-platelet sources, of which the vascular endothelium may be the most important. Switalska et al. (15) suggest on the basis of anecdotal observations that most plasma thrombospondin is derived from platelets, but do not present any data to this effect. They also claim on a quantitative basis that endothelial cells are unlikely to secrete thrombospondin into circulating blood under physiological conditions, but fail to take account of the likelihood that the kinetics of production differ *in vivo* and *in vitro*, that adult endothelial cells secrete much less thrombospondin in culture than the umbilical vein cells previously studied (16), and the unknown contribution of the microvasculature.

This demonstration of a background plasma thrombospondin concentration independent of the presence of platelets provides an explanation of the poor performance of thrombospondin as an indicator of platelet release in disease (3). Not only does any release have to be detected against this background, but in addition the contribution from non-platelet sources may itself vary in different clinical conditions. Furthermore, a significant proportion of the thrombospondin released from the  $\alpha$ -granule may not appear in the plasma, becoming associated with the platelet surface (15, 17) or the fibrin clot (18). These factors do not adequately account for the unsuitability of plasma thrombospondin levels as a marker of platelet activation.

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# Thrombospondin in Malignant and Non-malignant Breast Tissue

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**Abstract**—Cytosols of malignant breast tissue contained significantly higher levels of thrombospondin (TSP) and von Willebrand factor (vWF) than non-malignant breast. TSP and vWF content of human breast were significantly correlated whereas there was no correlation between TSP and the platelet-specific protein  $\beta$ -thromboglobulin ( $\beta$ TG). Whilst TSP in pre-menopausal breast cancer was slightly lower than in post-menopausal breast cancer, it did not correlate with oestrogen receptors (ER) or progesterone receptors (PR), but was negatively correlated with tissue-type plasminogen activator (tPA), an oestradiol-inducible enzyme. Secretion of TSP by MCF-7 cells was low and refractory to hormones. High levels of TSP appeared to be associated with the centre of the tumour mass. It is suggested that activation of the endothelium may be responsible, at least in part, for the high levels of TSP found in malignant breast tissue and could be a factor in the growth and spread of breast cancer.

## INTRODUCTION

THROMBOSPONDIN (TSP), a glycoprotein first described in platelets and subsequently in a variety of cell types, is incorporated into the extracellular matrix of some cells in culture where it may function as an adhesive protein (for reviews see Refs. [1-3]). As a component of the extracellular matrix, TSP has been shown to have an autocrine function, augmenting the mitogenic response of smooth muscle cells to epidermal growth factor [4].

TSP has also been implicated in tumour cell metastasis. For example it promotes the attachment and spreading of several human cancer cells *in vitro* [5-7] and a recent report shows that TSP potentiates tumour cell metastasis in mice [8]. Specific receptor sites for TSP have also been identified on the membrane of platelets, endothelial cells and a variety of human tumour cells [9]. As a result, TSP could be involved at several points in the metastatic process through potential interactions of malignant cells with extracellular matrix, endothelium or circulating platelets.

TSP is present in high concentrations in milk, other breast secretions and in some types of cyst

fluid [10]. We reported recently that TSP is a component of the aqueous phase of milk, that its level falls during the transition of colostrum to mature milk and also that malignant breast had a higher content of TSP than non-malignant breast [11]. The increased TSP levels observed in these studies did not reflect platelet activation since there were no parallel changes in the platelet-specific protein  $\beta$ -thromboglobulin ( $\beta$ TG). Tissue-type plasminogen activator (tPA), the major plasminogen activator (PA) in human milk, shows a similar pattern of secretion during early lactation as does TSP [12]. Increased levels of PA have also been reported in breast tumours [13] where they correlate with oestrogen receptors (ER) and progesterone receptors (PR) [14]. Moreover, production of PA by breast cells in culture is regulated by oestradiol and other steroid hormones [15-17].

Von Willebrand factor (vWF) is synthesized by endothelial cells and megakaryocytes and is contained in platelet  $\alpha$ -granules. vWF functions as a carrier protein for coagulation factor VIII and is thought to be involved in platelet-vessel wall interactions [18]. Indeed, vWF is incorporated into the extracellular matrix of cultured endothelial cells [19] where it may function as an adhesive protein. Although not specific for endothelial cells, vWF has been used as a marker of endothelial perturbation *in vitro* [20-22] and as a criterion for endothelial cell identification [23].

We have undertaken a more comprehensive study

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of TSP in the human breast and the factors which may be responsible for the high levels which are associated with malignancy. Our findings suggest that endothelial activation may be responsible, at least in part, for the elevated TSP in breast cancer and this may have a role in regulating the growth and spread of the malignant epithelium.

## MATERIALS AND METHODS

### Samples

Breast tissue cytosols from patients attending the Edinburgh breast clinic (101 breast carcinoma, nine benign mammary dysplasia, five fibroadenoma, one gynaecomastia, one normal) were prepared by homogenization of tissue in 20 mM Tris-HCl, pH 7.6, containing 0.25 M sucrose, 1 mM  $\text{CaCl}_2$ , 2 mM  $\text{MgCl}_2$ , 10 mM KCl at a dilution of either 1:5 (w/v) or 1:10 (w/v) using a Silverson homogenizer. They were centrifuged at 105,000 *g* at 4°C for 1 h and the supernatants stored at -20°C.

Eleven breast tissue cytosols from five patients with breast cancer were obtained at the time of mastectomy by snap-freezing of washed tissue samples followed by cryofragmentation using a mechanical dismembrator, and preparation of a supernatant from the tissue extract resuspended in buffered saline. They were classified according to whether the tissue had been taken from the centre of the tumour ( $n = 5$ ) or the surrounding non-malignant tissue ( $n = 6$ ).

### Assays

TSP,  $\beta\text{TG}$  and vWF were measured by radioimmunoassays as previously described [24-26]. The sensitivity of the radioimmunoassay for vWF was 22 ng/ml but it was not possible to specify a detection limit due to the varying protein concentrations of individual cytosols. tPA was measured by enzyme-linked immunosorbent assay [27] by Dr. P. Gaffney and the results have been published elsewhere [28].

ER was measured in low speed supernatants of breast tissue homogenates as previously reported [29]. PR was determined in the same high speed supernatant used for TSP measurement as described elsewhere [30].

Protein assays were performed by a dye-binding method (Bio-Rad Laboratories Ltd., Watford, U.K.) using a bovine serum albumin standard.

### Cell culture

Briefly, MCF-7 human breast cancer cells (subcultured from a stock obtained from Professor S.C. Brooks, Michigan Cancer Foundation) were grown on 10  $\text{cm}^2$  plastic culture wells (Costar, Cambridge, MA) in Eagle's MEM with Hank's BSS (Flow Laboratories, Irvine, U.K.) supplemented with NEAA, sodium pyruvate, L-glutamine, insulin, penicillin, streptomycin and 10% FCS (Gibco, Pais-

ley, U.K.). At confluence, the monolayer was washed extensively with basal MEM to remove serum components and then incubated with 2 ml/well serum-free medium according to Barnes and Sato [31] with or without the test substance. Culture supernatants were decanted after 48 h, centrifuged to remove cell debris and stored at -40°C prior to assay.

### Statistical analyses

Statistical analysis of differences between populations was performed by the Wilcoxon-Mann-Whitney rank sum test. Correlations between analytes were calculated by the Spearman rank correlation test.

## RESULTS

### TSP, vWF and $\beta\text{TG}$ in breast cytosols

A wide range of TSP levels was found in breast cytosols (Fig. 1). Highest levels were observed in breast cancers ( $n = 101$ , median = 317  $\mu\text{g}$  TSP/g cytosol protein, range = 17.4-23,400  $\mu\text{g}$  TSP/g cytosol protein) and lowest in benign breast ( $n = 15$ , median 21.7, range = 1.78-155). The difference between cancer and benign samples was

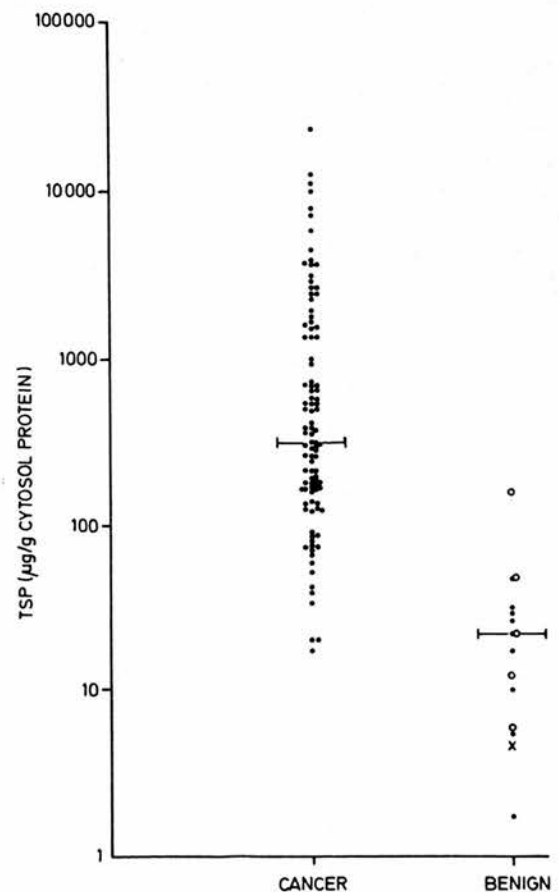


Fig. 1. TSP in breast cancer and benign breast tissue. Open circles represent fibroadenomas and one gynaecomastia is shown as a cross. Horizontal lines indicate median values. The difference between cancers and benigns was highly significant ( $P < 10^{-6}$ ).

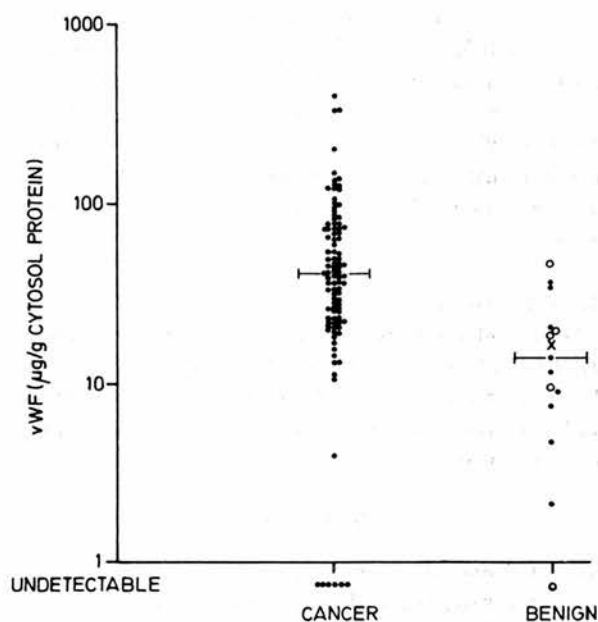


Fig. 2. vWF in breast cancer and benign breast tissue. Symbols as for Fig. 1. The difference between cancers and benigns was significant ( $P < 10^{-4}$ ).

highly significant ( $P < 10^{-6}$ ). TSP in fibroadenomas ( $n = 5$ , median = 21.7, range = 5.93–155) was not significantly different from other types of benign breast tissue ( $n = 10$ , median = 19.7, range = 1.78–47.6) and therefore all benigns were considered as a single group.

Measurement of vWF in breast cytosols revealed a narrower range of concentrations compared with TSP (Fig. 2), but higher levels of vWF were also found in cancers ( $n = 101$ , median = 40.8  $\mu\text{g}$  vWF/g cytosol protein, range = undetectable–392

$\mu\text{g}$  vWF/g cytosol protein) than in benigns ( $n = 15$ , median = 14.0, range = undetectable–46.0). The difference was significant ( $P < 10^{-4}$ ) although less marked than for TSP. A plot of TSP against vWF (Fig. 3) showed that they were significantly correlated ( $n = 116$ ,  $r_s = 0.484$ ;  $P < 0.001$ ).

$\beta\text{TG}$  was measured together with TSP in 20 breast cytosols (18 cancer, one benign, one normal) and no correlation was observed between the two ( $r_s = -0.365$ ;  $P > 0.10$ ) (Fig. 4).

#### TSP and indicators of hormonal status in breast cancer cytosols

Breast cancer cytosol TSP levels were compared in a group of 92 patients classified according to menopausal status (Fig. 5). TSP in breast cancer cytosols from pre-menopausal women ( $n = 32$ , median = 213.5  $\mu\text{g}$  TSP/g cytosol protein, range = 17.4–23400  $\mu\text{g}$  TSP/g cytosol protein) was slightly lower than that in cancers from post-menopausal women ( $n = 60$ , median = 367, range = 41.8–12800), the difference just reaching statistical significance ( $P = 0.043$ ).

TSP did not correlate with ER in breast cancers ( $n = 98$ ,  $r_s = -0.0075$ ;  $P > 0.20$ ) nor with PR ( $n = 33$ ,  $r_s = -0.146$ ;  $P > 0.20$ ) (Fig. 6a,b).

#### TSP secretion by MCF-7 human breast cancer cells

Levels of TSP in the supernatants from MCF-7 cells in serum-free culture for 48 h are shown in Table 1. Hormones (diluted into culture media from ethanolic solution) were added at concentrations previously shown to be maximally stimulatory for MCF-7 [32, 33]. No significant change in TSP secretion was observed (Wilcoxon–Mann–Whitney

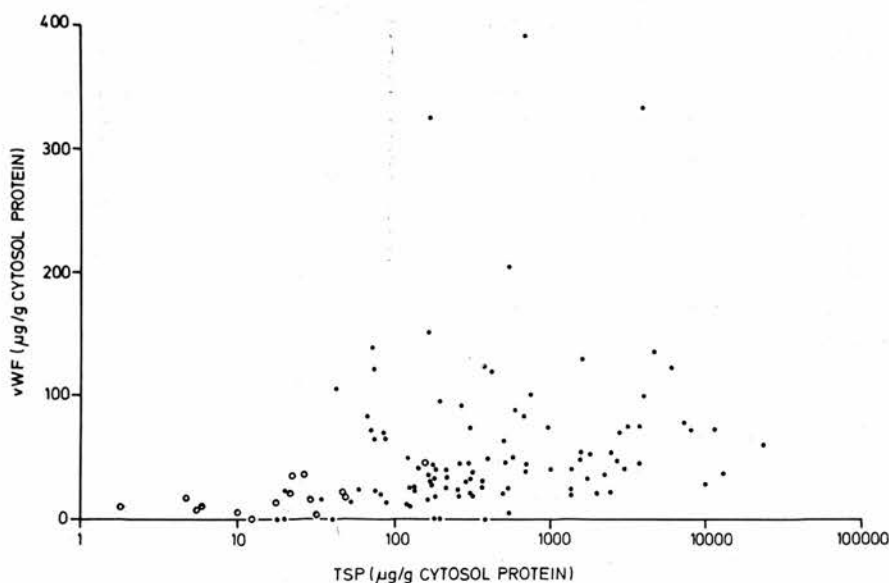


Fig. 3. Relationship between TSP and vWF in breast tissue. Closed circles represent cancers and benigns are shown as open circles. TSP and vWF were significantly correlated ( $r_s = 0.484$ ;  $P < 0.001$ ).

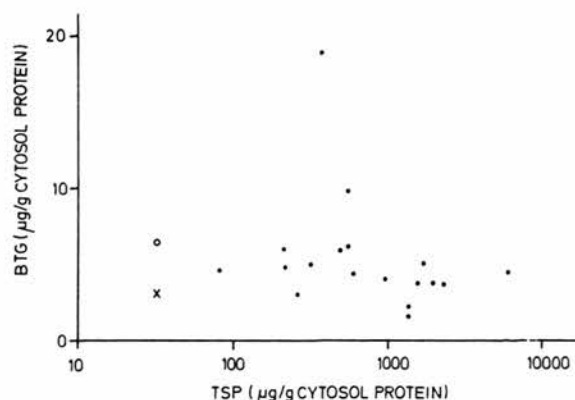


Fig. 4. Relationship between TSP and  $\beta$ TG in breast tissue. Symbols as for Fig. 3. One normal breast is shown as a cross. TSP and  $\beta$ TG were not correlated ( $r_s = -0.365$ ;  $P > 0.10$ ).

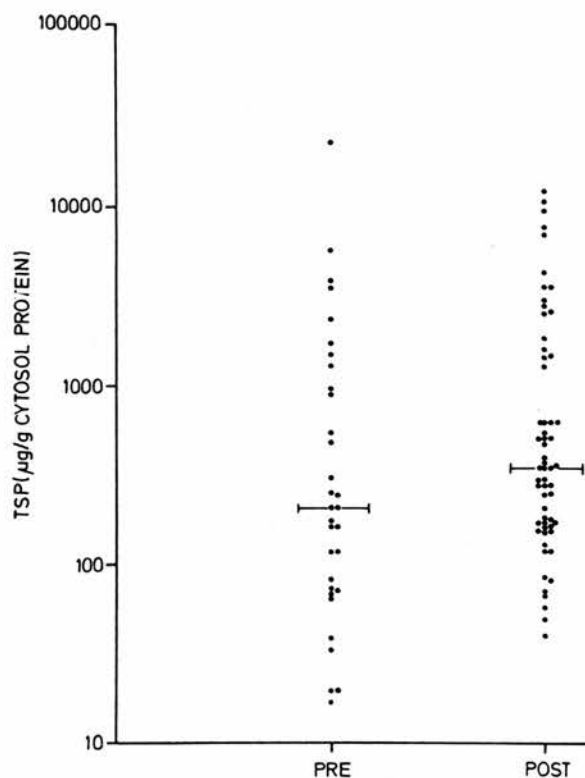


Fig. 5. Menopausal status and TSP in breast cancer tissue. Horizontal lines represent median values. The difference between pre- and post-menopausal cancers was marginally significant ( $P = 0.043$ ).

rank sum test) in the presence of any of the substances tested, and the rate of secretion was low in comparison with that reported for human endothelial cells [34].

#### *TSP and tPA in cytosols of tissue taken from different locations within cancerous breast*

Eleven breast tissue cytosols from five patients with breast cancer were measured for their TSP and tPA content. The tissue had been classified

Table 1a. Effect of hormones on TSP secretion by MCF-7 human breast cancer cells

	Secreted TSP 48 h
Control	88.0 (1.7)
17 $\beta$ -Oestradiol ( $10^{-8}$ M)	92.0 (6.9)
Testosterone ( $10^{-6}$ M)	95.7 (4.5)
5 $\alpha$ -Dihydrotestosterone ( $10^{-6}$ M)	97.3 (4.6)

Table 1b. Effect of EGF on TSP secretion by MCF-7 human breast cancer cells

	Secreted TSP 48 h
Control	83.3 (7.0)
EGF (1 ng/ml)	92.7 (3.2)
EGF (10 ng/ml)	90.0 (6.9)

Values represent mean (S.D.) TSP levels in ng/ml from triplicate experiments.

according to whether it was taken from the centre of the tumour mass ('centre') or from the surrounding non-malignant tissue ('surround'). Comparison of the TSP concentration in corresponding samples from centre and surround (Fig. 7) showed that in four out of the five patients, the centre ( $n = 5$ , median = 1120  $\mu$ g TSP/g cytosol protein, range = 31.8–4480  $\mu$ g TSP/g cytosol protein) contained much higher levels than the surround ( $n = 6$ , median = 50.9, range = 9.09–324). The difference between the two groups was significant ( $P = 0.041$ ).

On the other hand, in every case tPA in corresponding samples was lower in the centre ( $n = 5$ , median = 1.86  $\mu$ g tPA/g cytosol protein, range = 1.30–3.67  $\mu$ g tPA/g cytosol protein) than in the surround ( $n = 5$ , median = 7.18, range = 3.54–11.99) (Fig. 8). Again, the difference between the two groups was significant ( $P = 0.008$ ).

TSP and tPA were negatively correlated ( $n = 10$ ,  $r_s = -0.576$ ;  $P < 0.05$ ) (not shown).

## DISCUSSION

The elevated levels of TSP and vWF in malignant breast, and the correlation observed between these proteins in breast tissue suggest that they may have a common site of synthesis within the breast which is stimulated in malignant disease. vWF is synthesized by endothelial cells and megakaryocytes and is contained in platelet  $\alpha$ -granules [18]. It is incorporated into the subendothelial matrix and thought to be involved in interactions between platelets and vessel walls. TSP is also synthesized by endothelial cells and megakaryocytes, and is stored in platelet

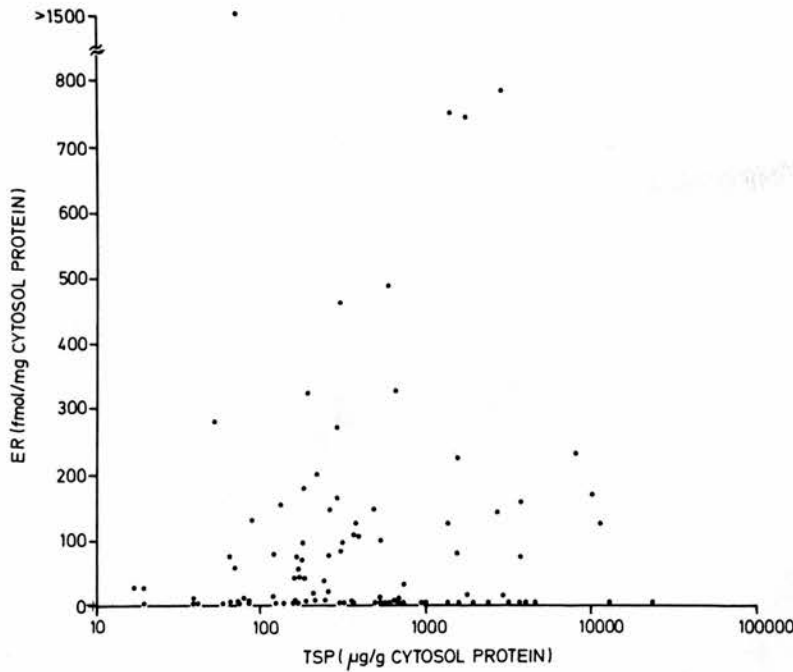


Fig. 6a. Relationship between TSP and ER in breast cancer tissue. TSP and ER were not correlated ( $r_s = -0.0075$ ;  $P > 0.20$ ).

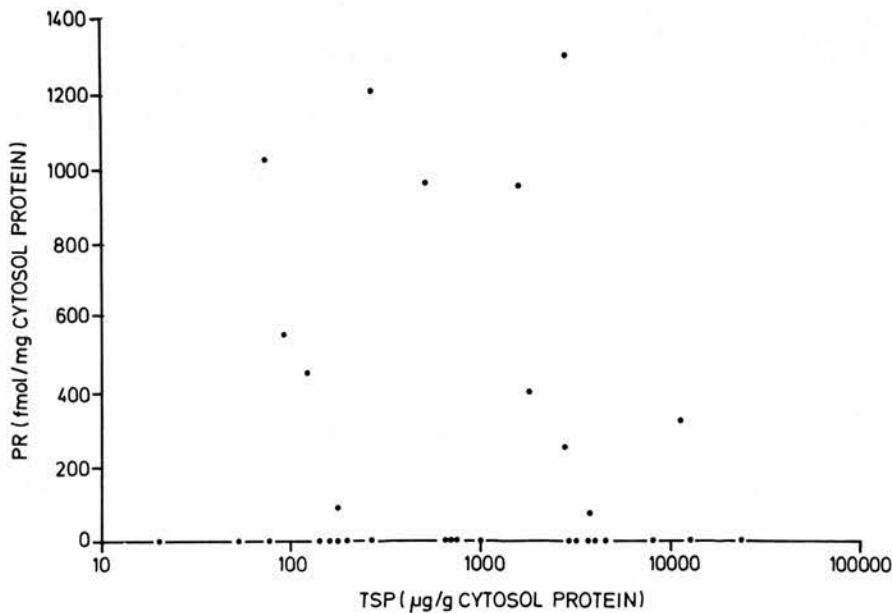


Fig. 6b. Relationship between TSP and PR in breast cancer tissue. TSP and PR were not correlated ( $r_s = -0.146$ ;  $P > 0.20$ ).

$\alpha$ -granules, but has also been described in a wide range of other cell types [1-3]. Since the platelet  $\alpha$ -granule protein  $\beta$ TG did not correlate with TSP in breast cytosols, it is very unlikely that platelets are the source of the high levels of TSP and vWF in malignant breast. Increased vascularity and/or stimulation of the endothelium could explain the higher TSP and vWF content of cancer tissue despite our finding that the difference between cancer and benign samples was much greater for

TSP than for vWF (the median cytosolic vWF of malignant breast was three times higher than non-malignant breast, whereas for TSP it was 15 times higher). TSP and vWF have been shown to be located in different subcellular compartments in endothelial cells where their secretion is separately controlled [35]. However, it is possible that another source within the breast, in addition to the endothelium, may be contributing to the very high level of TSP observed in some breast cancers.



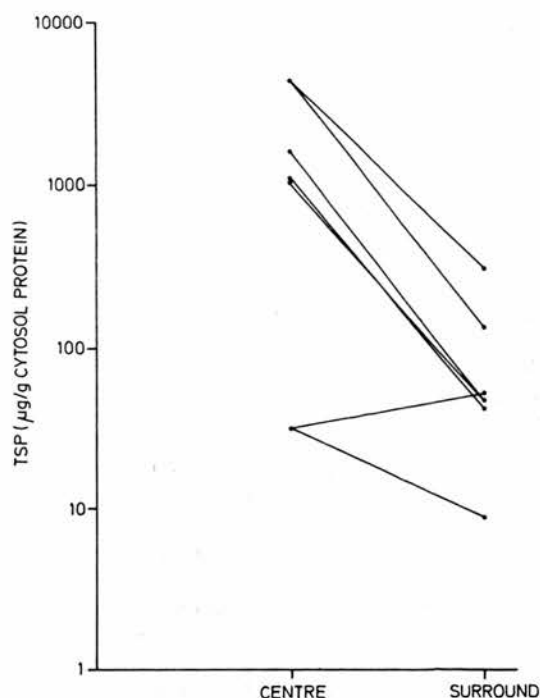


Fig. 7. TSP in breast tissue from different locations within cancerous breast. Lines connect corresponding samples taken from the centre of the tumour mass ('centre') and the surrounding non-malignant tissue ('surround') of the cancerous breasts of five patients. The difference between the two groups was significant ( $P = 0.041$ ).

The growth of certain breast cancers is hormonally mediated. Approximately 30% of breast cancers respond to hormone treatment and most of these are ER positive [36]. Since oestrogens are so closely implicated in breast cancer, it was of interest to investigate whether the high levels of TSP in malignant breast were related to any of the known indicators of hormonal status. A slight difference was observed in the TSP content of breast cancers from pre- and post-menopausal women, but no correlation was found between TSP and ER or PR. TSP was secreted only in small amounts by cultured human breast cancer cells in a manner refractory to steroid hormones and epidermal growth factor, and therefore it seems unlikely that synthesis of TSP by malignant epithelial cells regulated by oestrogens is involved in causing the elevated levels found in breast cancer.

Further evidence for the specific elevation of TSP in malignant breast came from the measurement of TSP in tissue from different sites within the cancer-

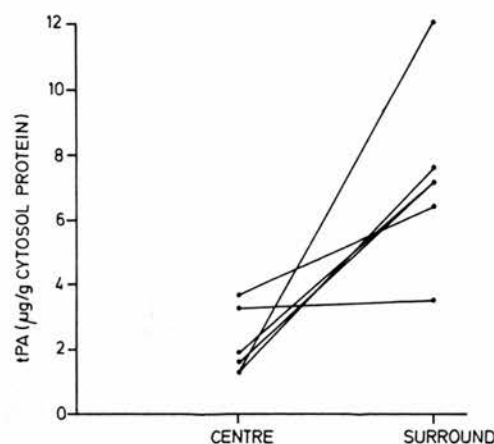


Fig. 8. tPA in breast tissue from different locations within cancerous breast. Lines connect corresponding samples as for Fig. 7. The difference between the two groups was significant ( $P = 0.008$ ).

ous breasts of a group of patients. Very high levels of TSP ( $>500 \mu\text{g/g}$  cytosol protein) were only seen in tissue taken from the centre of the tumour mass; levels in tissue taken from elsewhere within the same breast were comparable to those found in benign breast.

It was interesting that measurement of tPA in these samples showed the opposite trend. Whilst elevated PA levels in breast cancer have been reported [13], some tumours contain predominantly urokinase-type plasminogen activator (UKPA) and others predominantly tPA [37] which makes early studies in which only total PA was measured difficult to interpret. However, it has been established that tPA, rather than UKPA, is induced by oestradiol [14, 38] and our finding that TSP is inversely correlated with tPA suggests that TSP and tPA are not coordinately expressed by the malignant epithelium.

In order to define the exact source and role of TSP in the breast further studies are required. To this end we are currently using immunocytochemical techniques for the localization of TSP *in situ* and attempting to characterize TSP isolated from human breast.

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